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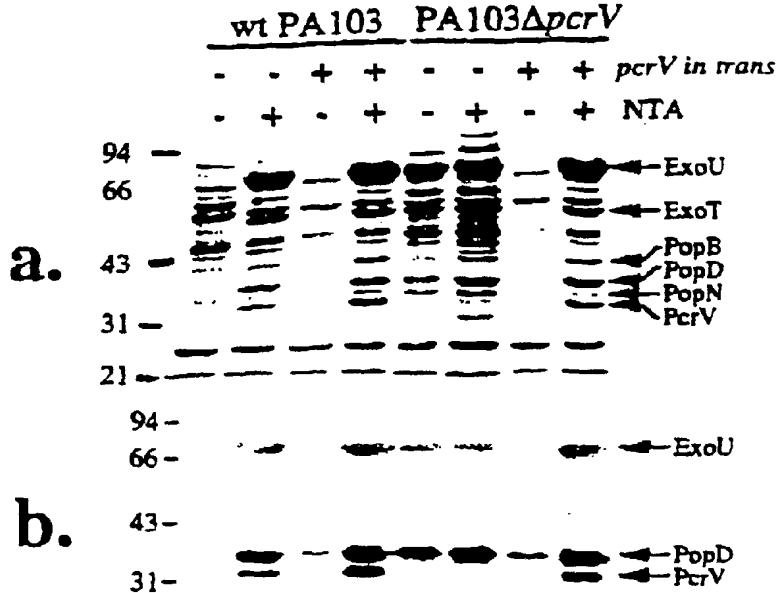
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(54) Title: METHOD AND COMPOSITIONS FOR IMMUNIZATION WITH THE PSEUDOMONAS V ANTIGEN



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(57) Abstract: A method of inhibiting, moderating or diagnosing *Pseudomonas aeruginosa* infection is disclosed. In one embodiment, this method comprises inoculating a patient with an effective amount of PcrV antigen.



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METHOD AND COMPOSITIONS FOR IMMUNIZATION WITH THE  
PSEUDOMONAS V ANTIGEN

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RESEARCH OR DEVELOPMENT

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BACKGROUND OF THE INVENTION

*Pseudomonas aeruginosa* is an opportunistic bacterial pathogen that is capable of causing fatal acute lung infections in critically ill individuals (1).

10 The ability of the bacterium to damage the lung epithelium has been linked with the expression of toxins that are directly injected into eukaryotic cells via a type III-mediated secretion and translocation mechanism (2, 3).

The proteins encoded by the *P. aeruginosa* type III secretion and translocation apparatus demonstrate a high level of amino acid identity with 15 members of the *Yersinia* Yop regulon (4-6). Of all the type III systems discovered in Gram-negative bacteria, only *P. aeruginosa* possesses a homologue to the *Yersinia* V antigen, PcrV (see 6 for review of type III systems). Homologous proteins to the secretion and translocation apparatus are encoded by both plant and animal pathogenic bacteria. These organisms 20 include human pathogens such as *Salmonella typhimurium*, *Shigella flexneri*, *Enteropathogenic E. coli*, *Chlamydia spp.*, and plant pathogens such as *Xanthomonas campestris*, *Pseudomonas syringae*, *Erwinia amylovora* and *Ralstonia solanacearum*. However, only *P. aeruginosa* and *Yersinia* encode the V antigen.

25 Yahr, et al., 1997, discloses the sequence of the operon encoding PcrV and compares the sequence to the LcrV protein. Thus, the amino acid

sequence of PcrV is known and is available under accession number AF010149 of GenBank.

## SUMMARY OF THE INVENTION

The present invention involves methods and compositions developed  
5 from our observation that the *Pseudomonas* V antigen can be used to protect  
animals from a lethal lung infection.

In one embodiment, the present invention is a method of inhibiting  
*Pseudomonas* infection comprising inoculating a patient with an effective  
amount of PcrV antigen. In another embodiment, DNA encoding PcrV is  
10 used as a gene vaccine.

In one preferred embodiment, the antigen is expressed as a recombinant protein and used to immunize patients at risk.

Preferably, the patient is completely protected from infection.

In another embodiment, the DNA encoding PcrV (called *pcrV*) or a  
15 DNA fragment may be used diagnostically to detect *P. aeruginosa* infection.

In another embodiment, the recombinant protein (rPcrV) is used  
diagnostically to detect antibodies from patients. Patient antibody response  
to PcrV may be associated with prognosis. Therefore, in this embodiment the  
recombinant protein is used as a prognostic indicator by measuring the  
20 patient's antibody titer.

The present invention also provides a method for inhibiting a  
*Pseudomonas* infection in an individual by contacting the individual with an  
effective amount of a PcrV inhibitor, in particular with a PcrV antibody,  
antibody derivative or fragment, or antibody mimic. PcrV antibodies, antibody  
25 derivatives and antibody fragments are also provided.

It is an object of the present invention to actively and passively  
immunize a patient against *Pseudomonas* infection.

It is another object of the present invention to diagnostically detect the *P. aeruginosa* infection.

It is another object of the present invention to diagnostically detect antibodies from *Pseudomonas* patients.

5 Other objects, features and advantages of the present invention will become apparent to one of skill in the art after review of the specification, claims and drawings.

#### DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B are a stained gel (Fig. 1A) and Western blot (Fig. 1B)  
10 illustrating the phenotypic analysis of PA103ΔpcrV.

Figs. 2A and 2B are a graph (Fig. 2A) and set of bar graphs (Fig. 2B)  
illustrating the survival and lung injury of *P. aeruginosa* parental and mutant  
strains.

Figs. 3A and 3B are a graph (Fig. 3A) and a set of bar graphs (Fig. 3B)  
15 illustrating the effect of immunization on survival, lung injury, and bacterial  
colonization.

Fig. 4 is a graph of the number of animals surviving a challenge with 5  
 $\times 10^5$  CFU/mouse of strain PA103 after passive administration of polyclonal  
IgG specific for PcrV, ExoU, PopD or control IgG from an unimmunized  
20 animal.

Fig. 5 is a graph (Fig. 5A) and a set of bar graphs (Fig. 5B) illustrating  
survival and protection from lung injury by concomitant administration of IgG  
to different bacterial antigens and bacterial challenge. One-way ANOVA for  
lung injury, p=0.026, and lung edema, p<0.0005.

25 Figs. 6A and B are printouts of SEQ ID NOS:1 and 2 with additional  
explanatory information. Fig. 6A is SEQ ID NO:1. Fig. 6B is SEQ ID NO:2.

Fig. 7 is a printout of SEQ ID NOs:3 and 4 with additional explanatory information.

Fig. 8 is a synthetic recombinant single chain antibody (SCFV-M166) (SEQ ID NOs:5 and 6).

5

## DESCRIPTION OF THE INVENTION

We disclose herein that PcrV has a novel regulatory effect on expression of the type III secreted products, is involved in the translocation of type III toxins, and is the first antigen that protects against lung injury induced by *P. aeruginosa* infection. Vaccination against PcrV prior to the airspace instillation of anti-PcrV IgG not only ensured the survival of challenged animals but also decreased lung inflammation and injury caused by the bacteria.  
10

LcrV, or the V antigen, is a multifunctional protein that regulates secretion/translocation of the Yop effector proteins and plays an extracellular role in pathogenesis by altering the host cytokine response to *Yersinia* infection (7-11). The only known homologue of this critical pathogenic factor is an extracellular protein encoded by *P. aeruginosa*, termed PcrV.  
15

One embodiment of the present invention is a method of moderating or inhibiting a *Pseudomonas* infection by immunizing a patient with an effective amount of the PcrV antigen. By "effective amount" we mean an amount of PcrV antigen effective to show some moderation or inhibition of *Pseudomonas* infection as compared to control subjects or animals who have not been treated with the antigen.  
20

By "moderating" we mean that infection is inhibited by at least fifty percent compared to a non-immunized animal. Preferably, infection is completely prevented. A quantitative assessment of infection would preferably include the examination of the amount of bacteria in the  
25

bloodstream or pleural fluids and/or an examination of lung injury parameters. For example, the absence of bacteria in the bloodstream or pleural fluids would indicate prevention of infection. A reduction in lung injury parameters would indicate that infection is moderated.

5        Infection could be quantitatively assessed by several other clinical indicators, including the reduction of bacterial load in the sputum, blood or pleural fluids, reduction in the size of the infiltrate, oxygenation improvement, reduction in the length of time on mechanical ventilation, reduction in fever and reduction in white blood cell count.

10       By "PcrV antigen" we mean that portion or fragment of the PcrV protein that is necessary to invoke an immune response which prevents or moderates infection. We have used the full-length PcrV protein as an antigen to induce protection. Additionally, we have mapped the protective epitope to the fragment comprising amino acids 144-257 of PcrV. To define the epitope, 15      monoclonal antibodies that protected against infection and cytotoxicity were tested for binding to progressively smaller forms of recombinant PcrV. (By "recombinant PcrV" or "rPcrV" we mean the protein produced from a PcrV gene that has been placed in a non-native host.) This protection localized the region.

20       The PcrV antigen may be most easily obtained by the method we used, commercially available bacterial expression plasmid pet16b from Novagen. The pcrV gene was first cloned from the *P. aeruginosa* chromosome as part of an operon. The coding region was amplified and inserted into two different vectors. One vector is for expression from *P. aeruginosa* as shown in Fig. 1. This is a vector from Herbert Schweizer 25      (reference 19) which we modified to contain an appropriate promoter sequence such that PcrV expression is coordinately regulated with the rest of

the delivery and intoxication apparatus of the bacterium. The second plasmid, pET16b, is for expression and purification purposes from *E. coli*.

The advantage of this system is that we do not have to worry about contaminating *P. aeruginosa* proteins, the protein is produced in great abundance, and there is a one-step purification process. In this situation the PcrV coding region is amplified to be cloned in frame with a histidine tag provided on the pET16b vector. The multiple histidine residues fused to the amino terminus of PcrV allow affinity chromatography using a nickel-NTA column. Therefore, a preferable PcrV antigen is a recombinant version of the natural PcrV protein.

Immunization may be done systemically or intranasally. Immunization of these individuals would preferably start during normal vaccination procedures for other childhood diseases. We would predict long-lived protection with booster doses probably around ages 5 and 10.

In another embodiment, one would use DNA encoding the PcrV protein or the complement of this DNA to diagnostically detect *P. aeruginosa* infection. One would obtain the DNA sequence of the PcrV antigen at GenBank AF010149. The coding region for PcrV is at nucleotides 626-1510. One may also choose to use a fragment of this coding region or complement of this fragment. A successful probe is one that will hybridize specifically to the PcrV DNA and not to other regions.

One would preferably use a hybridization probe of at least 40 continuous nucleotides within the antigen sequence or two primers of at least 25 continuous nucleotides within the sequence. One skilled in the art would appreciate that many standard forms of nucleic acid diagnostic techniques would be suitable, for example, hybridization of the single-stranded 40 nucleotide probe to DNA or RNA extracted from a patient's sputum. In another example, patient's sputum would be used as a source for bacterial

DNA or RNA to serve as a template for the PCR or RT-PCR reaction, respectively.

One would also determine *Pseudomonas aeruginosa* infection in an individual by contacting a sample obtained from the individual with an antibody specific for PcrV and correlating enhanced antibody binding as compared with a control sample with *Pseudomonas aeruginosa* infection in the individual.

In an additional embodiment, the DNA encoding PcrV is used as a gene vaccine using standard molecular biological methods. For example, one could review the following references for techniques known to those of skill in the art: Davis, H.L., *et al.*, "DNA vaccine for hepatitis B: Evidence for immunogenicity in chimpanzees and comparison with other vaccines," *Proc. Natl. Acad. Sci.* 93:7213-7218, 1996; Barry, M.A., *et al.*, "Protection against mycoplasma infection using expression-library immunization," *Nature* 377:632-635, 1995; Xiang, Z.Q., *et al.*, "Immune responses to nucleic acid vaccines to rabies virus," *Virology* 209:569-579, 1995. By "effective amount" of a gene vaccine, we mean an amount of vaccine effective to moderate or eliminate *Pseudomonas* infection or *Pseudomonas* infection symptoms.

The protein or antigen could also be used diagnostically to detect antibodies in patients and, thus, predict the patient's infection status. One would preferably contact a sample obtained from an individual suspected of *Pseudomonas* infection with the PcrV protein or fragment thereof and detect protein/antibody binding. One would then correlate enhanced antibody binding (as compared with a control sample) with *Pseudomonas aeruginosa* infection in the individual. One could also use the PcrV antibody or antibody fragments therapeutically.

In another embodiment, the invention is the use of the antibody sequence (which we report below and in SEQ ID NOs:1-4) to produce

recombinant single chain antibodies that may block PcrV and could also utilize the sequence in gene delivery experiments, where one would deliver eukaryotic vectors that will then lead to the production of single chain antibodies in animals for prolonged periods. The sequence could also be  
5 utilized to humanize the murine monoclonal antibody to produce a product that can be utilized in human patient care.

Once the antibody is safe for human use, one could: (a) administer it systemically and (b) administer it into the lungs as either a preventative treatment or as a therapy. In order to use the PcrV antibody in humans, the  
10 antibody is preferably "humanized". In general, once the monoclonal antibody is obtained the heavy and light chain variable regions are cloned. These cloned fragments are then inserted into a human antibody backbone (constant regions). Thus, we can control the class of antibody (IgG, IgA, etc.) in addition to providing the binding specificity.

15 For use in the present invention, the PcrV antibody may be a monoclonal antibody or polyclonal. The antibodies may be human or humanized, particularly for therapeutic applications. Antibody fragments or derivatives, such as an Fab, F(ab')<sub>2</sub> or Fv, may also be used. Single-chain antibodies, for example as described in Huston, *et al.* (*Int. Rev. Immunol.*  
20 10:195-217, 1993) may also find use in the methods described herein. By "effective amount" of the PcrV antibody or antibody fragment we mean an amount sufficient to moderate or eliminate *Pseudomonas* infection or infection symptoms.

Preferably, human or humanized monoclonal or polyclonal antibodies  
25 to PcrV are administered to prevent or treat infections with *P. aeruginosa*. In patients at high risk for *P. aeruginosa* infection, antibodies could be administered for prevention of infection. In addition, antibodies may be administered after the onset of infection to treat the infection. In this case,

antibodies can be administered alone or in combination with antibiotics.

Administration of antibodies in conjunction with antibiotics may allow the administration of shorter courses or lower doses of antibiotics, thereby decreasing the risk of emergence of antibiotic-resistant organisms.

- 5        We envision at least three types of hypothetical patients: (1) A healthy individual at risk of serious injury or burn (fire fighter, military personnel, police) would be immunized with the vaccine by a methodology (either injection or intranasal) that would give long-lived protection. A booster would be given on admission (intramuscular injection) to the hospital after injury. (2)  
10      A patient who is being subjected to mechanical ventilation. (3) A patient who has been genetically diagnosed with cystic fibrosis.

In addition to PcrV antibodies and antibody fragments, small molecule peptidomimetics or non-peptide mimetics can be designed to mimic the action of the PcrV antibodies in inhibiting or modulating *Pseudomonas* infection,  
15      presumably by interfering with the action of PcrV. Methods for designing such small molecule mimics are well known (see, for example, Ripka and Rich, Curr. Opin. Chem. Biol. 2:441-452, 1998; Huang, et al., Biopolymers 43:367-382, 1997; al-Obeidi, et al., Mol. Biotechnol. 9:205-223, 1998). Small molecule inhibitors that are designed based on the PcrV antibody may be  
20      screened for the ability to interfere with the PcrV-PcrV antibody binding interaction. Candidate small molecules exhibiting activity in such an assay may be optimized by methods that are well known in the art, including for example, *in vitro* screening assays, and further refined in *in vivo* assays for inhibition or modulation of *Pseudomonas* infection by any of the methods  
25      described herein or as are well known in the art. Such small molecule inhibitors of PcrV action should be useful in the present method for inhibiting or modulating a *Pseudomonas* infection.

In another aspect of the present invention, PcrV protein may be used to identify a PcrV receptor which may be present in the host cells, particularly in human cells, more particularly in human epithelial cells or macrophages. Identification of a PcrV receptor allows for the screening of small molecule 5 libraries, for example combinatorial libraries, for candidates that interfere with PcrV binding. Such molecules may also be useful in a method to inhibit or modulate a *Pseudomonas* infection.

Our first attempts at receptor identification will be to use PcrV in pull-down experiments. PcrV will be fused to glutathione S-transferase (GST) and 10 attached to column matrix for affinity chromatography of solubilized cellular extracts. Proteins binding specifically to PcrV will be eluted and subjected to amino terminal sequencing for identification. In parallel experiments PcrV will be subjected to yeast two-hybrid analysis. In this case PcrV is fused in frame with the DNA binding domain of Gal4. Once the clone is obtained it will be 15 transformed into a suitable yeast host strain. The yeast strain containing the Gal4PcrV construct will be transformed with a Hela cell cDNA bank cloned in frame with the Gal4 activation domain. Double transformants that complement the ability to utilize histidine and produce beta galactosidase 20 (proteins that interact with PcrV) will be analyzed genetically and at the nucleotide sequence level. In case the receptor is a cellular glycolipid we will utilize an overlay technique where glycolipids are separated by thin-layer chromatography and then probed with radiolabeled bacteria. The binding to specific components will be monitored by autoradiography. Similarly, 25 epithelial and macrophage proteins will be separated by SDS-PAGE, blotted onto nitrocellulose and overlaid with radiolabeled bacteria or labeled PcrV. Again, the protein components to which the bacteria bind are then identified by autoradiography.

*Pseudomonas* species are known to infect a wide spectrum of hosts within the animal kingdom and even within the plant kingdom. As will be apparent to one of ordinary skill in the art, the compositions and methods disclosed herein may have use across a wide range of organisms in inhibiting or modulating diseases or conditions resulting from infection by a *Pseudomonas* species. The compositions and methods of the present invention are described herein particularly for application to *Pseudomonas aeruginosa* but it is well within the competence of one of ordinary skill in the art to apply the methods taught herein to other species.

10

## EXAMPLES

1. Role of PcrV in Cytotoxicity

To determine the role of PcrV in type III-mediated regulation/secretion, we constructed a nonpolar allele of PcrV and used the construct to replace the wild-type allele in *P. aeruginosa* strain PA103, a strain that is highly cytotoxic *in vitro* (3) and causes lung epithelial damage *in vivo* (12, 13). Cytotoxicity and lung injury are due to the production of a specific cytotoxin, ExoU (3).

PA103ΔpcrV was characterized by the expression of several extracellular products that are secreted by the *P. aeruginosa* type III system which include the ExoU cytotoxin (3), PcrV (5), and a protein required for the translocation of toxins, PopD (14). SDS-polyacrylamide gel electrophoresis of concentrated culture supernatants indicated that the parental strain, PA103 is induced for production and secretion of the type III proteins by growth in medium containing a chelator of calcium, nitrilotriacetic acid (NTA) (Fig. 1). When an expression clone encoding PcrV was provided in *trans* in the parental strain, extracellular protein production in response to the presence or absence of NTA is normal. PA103ΔpcrV exhibits a calcium blind phenotype;

extracellular protein production is strongly induced in both the presence and absence of NTA. These results suggest that the secretory system is fully functional but deregulated. This deregulated phenotype is in contrast to the calcium independent phenotype reported for an LcrV defective strain which  
5 fails to produce the extracellular Yops, grows at 37°C regardless of the presence or absence of calcium, and shows only partial induction of the Yops (7). Complementing PA103 $\Delta$ pqrV with a clone expressing wild-type PqrV restored normal regulation of extracellular protein production in response to NTA induction.

10 To test the contribution of PqrV to *P. aeruginosa* pathogenesis, two infection models were used. In an *in vitro* model the parental and several mutant derivative strains were compared for their ability to cause cytotoxicity in a CHO cell infection assay (3). The negative controls in this experiment included PA103 $popD::\Omega$ , which has been previously shown to be defective in  
15 the translocation of type III virulence determinants (14) and PA103 $\Delta$ exoU, which is non-cytotoxic due to the absence of ExoU production (3, 15).

After a 3 hour infection, CHO cells were unable to exclude trypan blue with the wild-type and  $\Delta$ pqrV strain complemented with a plasmid construct expressing PqrV. Staining did not occur when CHO cells were infected with  
20 the negative control strains or with PA103 $\Delta$ pqrV (data not shown). These results suggest that PqrV expression is required for cytotoxicity. Purified recombinant PqrV was not cytotoxic when added exogenously to tissue culture cells. Since secretion of the type III proteins required for translocation was unaffected by the deletion of *pqrV* (Fig. 1A and B), PA103 $\Delta$ pqrV appears  
25 to be defective in ExoU translocation.

Figs. 1A and 1B are a stained gel (Fig. 1A) and Western blot (Fig. 1B) illustrating the phenotypic analysis of PA103 $\Delta$ pqrV. The parental and  $\Delta$ pqrV derivatives, with and without a plasmid expressing PqrV in *trans*, were grown

in the absence or presence of the inducer of type III secretion in *P. aeruginosa*, nitrilotriacetic acid (NTA). The extracellular protein profile (Fig. 1A) was analyzed on a SDS-polyacrylamide gel (10%) stained with Coomassie blue. The migration of the *P. aeruginosa*-encoded type III proteins is indicated to the left and the migration of molecular weight markers is indicated on the right. Fig. 1B is a Western blot of a duplicate gel using antibodies specific for ExoU, PcrV, and PopD and  $^{125}\text{I}$ -Protein A to detect bound IgG.

Wild-type and mutant *P. aeruginosa* strains were tested in an acute lung infection model using low and high challenge doses of bacteria. Survival measurements indicated that PcrV and PopD were required to induce a lethal infection (Fig. 2A). In experiments utilizing three independent measurements of lung injury (the flux of labeled albumin from the airspaces of the lung to the bloodstream, the flux of labeled albumin from the airspaces of the lung to the pleural fluids, and the wet/dry ratio, which measures lung edema) the degree of injury caused by PA103 $\Delta$ pcrV, the vector control strain (PA103 $\Delta$ pcrVpUCP18), and PA103popD::Ω were no different than the uninfected control animals (Fig. 2B). Complementation of PA103 $\Delta$ pcrV with pcrV in trans restored lung injury levels to those measured with the parental strain, PA103. Taken together these data indicate that PcrV expression is required for virulence of *P. aeruginosa* in the acute lung infection model and that part of the function of PcrV appears to be linked to the ability to translocate type III effector proteins into eukaryotic cells.

Figs. 2A and 2B are a graph (Fig. 2A) and set of bar graphs (Fig. 2B) illustrating the survival and lung injury of *P. aeruginosa* parental and mutant strains. Referring to Fig. 2A, mice were challenged with  $5 \times 10^5$  cfu of each of the indicated strains and survival was monitored for one week. Referring to Fig. 2B, lung injury was assessed by the flux of labeled albumin from the

airspaces of the lung to the blood (lung epithelial injury), to the pleural fluid (pleural fluid) or by measuring the wet/dry ratio (lung edema). Two bacterial infectious doses were used as denoted by the solid and striped bars.

Significant differences (\*p<0.001) between control and test groups was

5 determined by one-way ANOVA and Dunnet multiple comparison tests. The following abbreviations were used: PA103, parental wild-type strain;  $\Delta pcrV$ , PA103 $\Delta pcrV$ ;  $\Delta pcrVpUCPpcrV$ , PA103 $\Delta pcrV$  complemented with a plasmid expressing *PcrV*;  $\Delta pcrVpUCP$ , PA103 $\Delta pcrV$  with a vector control; *popD*::Ω, PA103*popD*::Ω, a translocation defective strain.

10 2. Immunization with PcrV

To determine whether immunization with PcrV protected animals from a lethal lung infection, recombinant PcrV (rPcrV) or ExoU (rExoU) were purified as histidine-tagged fusion proteins and used as antigens. Mice were immunized and subsequently challenged via their airspaces with a lethal dose 15 of strain PA103. When survival was measured, both vaccines protected the mice (Fig 3A). When lung injury was assessed, only PcrV vaccinated animals had significantly less epithelial damage and lung edema (Fig. 3B). Animals immunized with the PcrV vaccine also had significantly fewer bacteria in their lungs, suggesting that a blockade of the *Pseudomonas V* antigen may facilitate rapid clearance of bacteria from the lung, protecting the animals 20 from severe epithelial injury (Fig. 3B).

Figs. 3A and 3B are a graph (Fig. 3A) and a set of bar graphs (Fig. 3B) illustrating the effect of immunization on survival, lung injury, and bacterial colonization. Referring to Fig. 3A, mice were immunized (PcrV, n=10; ExoU, 25 n=5; control, n=10) as indicated and challenged with strain PA103 at  $5 \times 10^5$  CFU/animal. The percent of surviving animals was determined for one week; p<0.05 by the Mantel-Cox log rank test. Referring to Fig. 3B, lung injury assessment and bacterial colonization of vaccinated animals 4 hours after

installation of PA103. Lung epithelial injury, lung edema, and bacterial burden; PcrV, n=9; ExoU, n=4; and control, n=8. The final number of bacteria in the lung is indicated as the number on the Y axis x 10<sup>4</sup> CFU. Significant differences (\*) for lung injury (p<0.01), lung edema (p<0.05), and bacterial numbers (p<0.05) as determined by Dunnet multiple comparison test. One-way ANOVA for lung injury, p=0.0005; lung edema, p=0.0437; bacterial burden, p=0.0075.

To determine whether therapeutic intervention was possible, mice were passively immunized with preimmune rabbit IgG or rabbit IgG specific for rPcrV, rExoU, or rPopD one hour prior to airspace instillation of PA103 at a concentration of 5 x 10<sup>5</sup> CFU/mouse. Antibodies to rPcrV provided complete protection to a lethal infection (Fig. 4). Anti-rExoU IgG provided partial survival, which was significantly different from the administration of control IgG, although all the surviving animals appeared severely ill during the trial. Survival was not improved by the passive transfer of antibodies to another of the type III translocation proteins, PopD. From these results we conclude that antibodies to PcrV are highly protective in the acute lung infection model and that PcrV may be exposed on the bacterial surface or in a soluble form that is available for antibody-antigen interactions.

Fig. 4 is a graph of the number of animals surviving a challenge with 5 x 10<sup>5</sup> CFU/mouse of strain PA103. Animals were pretreated with 100 µg of immune IgG or control IgG from an unimmunized rabbit (rPcrV, preimmune serum). N=10 for each group; \*p<0.05 versus control group for treatment with anti-PcrV and anti-ExoU IgG preparations by Mantel-Cox log rank test.

If PcrV is accessible for neutralization, then concomitant administration of the bacterial inoculum with anti-rPcrV IgG should completely protect against lung injury and lethality. IgG preparations were mixed with the inoculum (10-fold higher dose than the lethal inoculum) prior to instillation of

the bacteria into the lung and survival was measured. Only anti-rPcrV IgG was protective against this extreme infection (Fig. 5A). Lung injury was measured in animals infected with the normal lethal dose of  $5 \times 10^5$  bacteria. The efflux of labeled albumin from the airspaces of the lung was only 3% more than uninfected controls (Fig. 5B) after co-administration of anti-rPcrV IgG. The decreased efflux of labeled protein from the lung to the pleural fluids was the same as the uninfected controls when anti-PcrV was included with the inoculum. Curiously lung edema, as measured by the wet/dry ratio, was significantly reduced by the addition of either anti-rPcrV or anti-rPopD. (Fig. 5B). Thus, the concomitant administration of anti-rPcrV IgG with the bacteria was even more effective in normalizing all the lung injury parameters than vaccination. These data support the accessibility of PcrV for antibody-mediated neutralization and document a clinically relevant decrease in lung injury; antibodies to PcrV may serve as therapeutic reagents in the treatment of severe nosocomial pneumonia caused by *Pseudomonas aeruginosa*.

Fig. 5 is a graph (Fig. 5A) and a set of bar graphs (Fig. 5B) illustrating survival and protection from lung injury by concomitant administration of IgG and bacterial challenge. IgG (5 µg) was mixed with either  $5 \times 10^6$  (for survival assays, n=10 per group) or  $5 \times 10^5$  (for the measurement of lung injury, n=4 to 6 animals per group) *P. aeruginosa* strain PA103. This mixture was instilled into the lungs and survival (Fig. 5A) or lung injury (Fig. 5B) was assessed. For survival, \*p<0.05 versus control IgG for anti-PcrV by the Mantel-Cox log rank test; for lung epithelial injury and lung edema \*p<0.05 versus control IgG by Dunnet multiple comparison test. One-way ANOVA for lung injury, p=0.026, and lung edema, p<0.0005.

In acute *P. aeruginosa* infections, the net effect of type III-mediated intoxication may be to promote the dissemination of the bacterium beyond the epithelium leading to infection of the pleural fluids, spleen, liver, and

bloodstream. Blood-borne infections with *P. aeruginosa* from either acute ventilator-associated pneumonia or from burn wound infections can result in a 40-80% mortality rate in spite of aggressive antibiotic treatment (16). PcrV must be a component of the type III translocation complex in *P. aeruginosa*,  
5 as mutants defective in the production of this protein are unable to intoxicate CHO cells or cause lung epithelial injury even though they are able to produce and secrete the type III effectors and proteins required for translocation. Unlike PopD, which is also necessary for translocation, PcrV is accessible for antibody-mediated neutralization suggesting that antibodies  
10 may be useful therapeutic agents in acute infections.

3. Methods for Examples 1 and 2

Construction of a nonpolar insertion in PcrV and complementation. A 5.0-kb *EcoRI-Nsil* restriction fragment encoding *pcrGVHpopBD* and flanking sequences was cloned into the allelic replacement vector pNOT19 (17). Two  
15 *NotI* sites (one within *pcrG* and one within *popB*) were removed from the inserted sequences by using the Sculptor mutagenesis system (Amersham). An internal *Ssfl* restriction fragment was deleted from *pcrV*, resulting in an in-frame deletion of residues 17-221 (pNOT $\Delta$ *pcrV*). To select for integration of the plasmid, a gene encoding tetracycline resistance (Tc $\Omega$ ) was cloned into  
20 the *HindIII* site of the vector (pNOT $\Omega\Delta$ *pcrV*). The MOB cassette (17) was added as a *NotI* fragment. Selection of merodiploids, resolution of plasmid sequences, and confirmation of allelic replacement was performed as previously described (18). A shuttle plasmid (pUCP, 19) was used to construct a clone to complement the *pcrV* deletion. The coding sequence for  
25 PcrV was amplified and cloned behind the control of the ExoS promoter region (20). The transcription of ExoS is coordinately regulated with the operons that control type III secretion and translocation in *P. aeruginosa* (2).

The nucleotide sequence was confirmed for each DNA construct involving site specific mutagenesis, PCR amplification, or in-frame deletion.

5       SDS-PAGE and Western blot analysis of secreted products. *P. aeruginosa* were grown under inducing (+NTA) or non-inducing conditions (-NTA) for expression of the type III secreted products (18). Cultures were harvested based on optical density measurements at 540 nm and supernatant fractions were concentrated by the addition of a saturated solution of ammonium sulfate to a final concentration of 55%. Each lane of an SDS-polyacrylamide gel (11%) was loaded with 3 µl of a 20-fold concentrated supernatant and stained with Coomassie blue. An identical gel was subjected to Western blot analysis as previously described (3-5) using a cocktail of rabbit antisera, which specifically recognizes ExoU, PopD, and PcrV. Protein A labeled with <sup>125</sup>I was used as a secondary reagent to identify bound IgG.

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15       Infection models and lung injury assessments. Chinese Hamster Ovary cells (CHO) were used in an *in vitro* infection model designed to measure cytotoxicity and type III translocation (21). Briefly, a bacterial inoculum was prepared in tissue culture medium without serum. CHO cells, which were propagated in serum containing medium, were washed and infected with various *P. aeruginosa* strains at a multiplicity of infection of 5:1. Cultures were incubated under tissue culture conditions for 3 hours (37°C, 20 5% CO<sub>2</sub>), washed, and stained with trypan blue. Permeability to the dye was determined from phase contrast photographs. Infection with the parental strain PA103, which expresses ExoU, results in trypan blue staining of approximately 80% of the monolayer after 3 hours of incubation and complete destruction of the monolayer at 4-5 hours of incubation. Mouse infections and assessment of lung injury was performed as previously described (16). Briefly, male 8- to 12-week old pathogen-free BALB/c mice were purchased

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from Simonsen Laboratories (Gilroy, CA) and housed in barrier conditions. The mice were briefly anesthetized with inhaled Metofane (methoxyflurane, Pitman-Moore, Mundelein, IL) and placed supine, at an angle of approximately 30°. Fifty microliters of the bacterial inoculum was instilled 5 slowly into the left lobe using a modified 24 gauge animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY) inserted into the trachea via the oropharynx. When lung injury assessments were measured, 0.5 µCi of <sup>131</sup>I-labeled human serum albumin (Merck-Frosst, Quebec, Canada), 0.05 µg of anhydrous Evans blue in ml of Ringer's lactate with 5% mouse albumin were 10 added to the instillate. After 4 hours of infection, the mice were anesthetized, blood was collected by a carotid arterial puncture and median sternotomies were performed. The lungs, pleural fluids, tracheas, oropharynxes, stomachs, and livers were harvested, and the radioactivity was measured. The percentage of radioactive albumin that left the instilled lungs and entered 15 the circulation or the pleural fluid was calculated by multiplying the counts measured in the terminal blood samples (per ml) times the blood volume (body weight X 0.07). The wet-dry ratios of the lungs were determined by adding 1 ml of water to the lungs and homogenizing the mixture. Homogenates were placed in preweighed aluminum pans and dried to 20 constant weight in an 80°C oven for three days. Lung homogenates were also sequentially diluted and plated on sheep blood agar for quantitative assessment of bacteria.

Production of rabbit antiserum to PcrV, PopD, and ExoU. rPcrV, rPopD, and rExoU were produced as histidine tagged fusion proteins in 25 pET16b and purified by nickel chromatography as previously described (22). Rabbits were injected intradermally (10 sites) with 300 µg of recombinant protein emulsified in Freund's complete adjuvant, boosted with antigen in Freund's incomplete adjuvant, and periodically bled at 7 day intervals. For

passive immunization, the IgG fraction was isolated using Protein A column chromatography (Pierce Chemicals, Rockford, IL). Mice were injected with 100 µg IgG (intraperitoneal injection) 1 hour before challenge with  $5 \times 10^5$  CFU of strain PA103. For active immunization with rPcrV and rExoU,

5 endotoxin was removed from protein preparations by extraction with 1% Triton X-114 (23). Following the extractions, Triton X-114 was removed by Sephadryl S-200 chromatography. All vaccine preparations contained less than 1 ng of endotoxin per 40 µg of recombinant protein as determined by using a limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD).

10 BALB/c mice were injected subcutaneously with 10 µg of recombinant proteins in Freund's complete adjuvant. At day 30 the mice were boosted with an additional 10 µg of antigen in Freund's incomplete adjuvant. On day 51 the mice were challenged by instillation of *P. aeruginosa* into their left lungs.

15 4. Synthesis of Monoclonal Antibodies

Mice were immunized with 10 µg of purified, LPS-free, recombinant PcrV in Freund's complete adjuvant and boosted two weeks later with the same dose of antigen emulsified in Freund's incomplete adjuvant. Immunizations were performed subcutaneously. Spleens were harvested

20 from mice one week after booster doses of PcrV in Freund's incomplete adjuvant.

A single spleen was placed in 5 ml of tissue culture medium without serum, cut into pieces and gently homogenized. Large pieces of tissue were allowed to settle from the homogenate and the supernatant, single-cell suspension was removed and subjected to centrifugation at 1200 rpm for 10 minutes. The pelleted cells were resuspended in 10 ml of a solution to lyse red blood cells for 5 minutes and subsequently underlaid with 10 ml of fetal bovine serum. The material was centrifuged at 1200 rpm for 8 minutes, the

supernatant was discarded and the cells were suspended in 30 ml of medium.

Spleenic cells and myeloma cells (P3x63Ag8.653) were harvested by centrifugation at 1200 rpm for 10 minutes, and each pellet was separately 5 suspended in 10 ml of tissue culture medium.  $10^8$  spleen cells and  $2 \times 10^7$  myeloma cells were mixed and pelleted together by centrifugation at 1200 rpm for 6 minutes. The supernatant was removed by aspiration and 1 ml of 35% polyethylene glycol (PEG) was added. The cells were suspended in this solution gently and centrifuged at 1000 rpm for 3 minutes. In some 10 experiments centrifugation was eliminated.

Exactly 8 minutes after the addition of PEG, 25 ml of medium was added and the cells were gently resuspended. Following a 5 minute 1200 rpm centrifugation step, the cell pellet was suspended at a density of  $1 \times 10^6$  per ml in 30% conditioned medium and 70% complete medium (with serum). 15 The cells were incubated overnight at 37°C. The next day the cells were harvested by centrifugation and suspended in 200 ml of 30% conditioned medium and 70% complete medium with hypoxanthine, aminopterin and thymidine (HAT).

Approximately 0.2 ml of this cell suspension was added per well to ten 20 96-well plates (12 ml per 96 well plate). The density of the remaining cells was adjusted to  $2.5 \times 10^5$  per ml and the cells were plated in the 96 well format. Plates were screened microscopically for single colonies and supernatants were subsequently tested for antibody production by enzyme-linked immunosorbent assay using recombinant PcrV as the antigen. Clones 25 producing antibodies reactive to PcrV were subcultured to larger culture dishes and then isotyping.

The binding of antibodies was tested in an enzyme linked immunosorbent assay using recombinant PcrV as the antigen (histidine-

tagged protein) coating the wells. Monoclonal antibodies were also tested in Western blot reactions using a *P. aeruginosa* supernatant containing native PcrV without the histidine tag.

5. Identification of PcrV Antigen

We obtained about three hundred cell lines producing antibodies that bound the tagged PcrV. These initial cell lines were preserved in liquid nitrogen for safekeeping. All cell lines were passaged to isolate stable clones. In conjunction with isolating stable clones we developed an *in vitro* assay as a correlate for protection against intoxication in animal infection models.

The hybridomas that were stable to passage and still produced antibodies reactive to PcrV in ELISA (approximately 80 cell lines) were subsequently tested in a Fluorescence Activated Cell Sorter using the following techniques and assumptions: We reasoned that if antibody is blocking the type III intoxication system, then in the presence of a monoclonal that blocks, fewer cells will be killed by our toxins. We exposed cells to each of the 80 monoclonal antibodies, added toxic bacteria, incubated, and then added a dye that is only permeable to dead cell DNA (propidium iodide). Excess dye was washed away and the cells were harvested, fixed, and analyzed by FACS. Dead cells would be fluorescent since the dye leaked in and stained DNA in the nucleus.

We found that if the cells were incubated with rabbit polyclonal anti-PcrV, mouse polyclonal anti-PcrV, or mab166 and bacteria, fewer cells died than in controls with irrelevant polyclonal antibody (anti-PopD) or the other 78 monoclonal antibodies.

Mab 166 was specifically found to bind to the bacterially encoded type III-secreted factor termed PcrV. PcrV mediates the interaction of *P. aeruginosa* and lung cells to facilitate the translocation of bacterial toxins that cause cellular death. This reaction is postulated to eliminate lung cells that are involved in the innate immune response to *P. aeruginosa*. The killing of these cells leaves the host epithelium open for *P. aeruginosa* colonization.

and spread to the pleural fluids and bloodstream. *P. aeruginosa*-encoded antibiotic resistance makes effective treatment unlikely once the bacteria have entered the bloodstream.

The protection afforded by mab 166 pre- and post-bacterial instillation  
5 in animal models of acute lung infection with *P. aeruginosa* is significant. To design antibody treatment modalities for intervention in human *P. aeruginosa* infections it will be necessary to produce either a human monoclonal antibody or to immunize at risk patients with the protective epitope of PcrV defined by mab 166. The goal of the work described below is to define the amino acid  
10 sequence of PcrV bound by mab 166.

### Results

We used a molecular genetic approach to define the amino acid residues bound by mab 166. PcrV possesses 294 amino acids. The approach consisted of deleting parts of the molecule at the nucleotide  
15 sequence level using the polymerase chain reaction. Each product was cloned into a protein expression vector in frame with a gene encoding the glutathione S transferase protein. This strategy ensured that deletions encoding small numbers of PcrV amino acids could be detected using Western or dot blot techniques. Control bacterial lysates encoding only  
20 glutathione S transferase showed no reactivity to either our anti-PcrV polyclonal or mab 166 monoclonal antibody.

A total of 66 (with one full-length PcrV expression plasmid) clones were constructed, expressed, and tested for reactivity to rabbit polyclonal anti-PcrV antisera. All but one clone bound to anti-PcrV rabbit antibody  
25 verifying that the expressed proteins were in-frame with PcrV. The one non-reactive clone was eliminated from the analysis. None of the C-terminal deletions ( $n = 5$  constructs) bound mab 166 suggesting that the epitope was in the C-terminal half of the protein. Only one of the N-terminal truncation

proteins (n = 8 constructs) encoding PcrV amino acids (aa) 139-294 bound to mab 166. This experiment verified our hypothesis that the mab 166 epitope was encoded by the carboxyl terminal half of the protein. The remaining 51 constructs encoded various internal deletions of the molecule. Binding analysis tabulated in Table 1, below, demonstrated that the smallest epitope recognized by mab 166 consists of aa 144-257 of PcrV.

TABLE 1

| <u>PcrV Epitope Mapping</u>                                |  |  |   |
|--|--|--|---|
| All proteins are amino-terminal tagged GST-PcrV truncates. |  |  |   |
|  | <u>Amino Acids</u>   | <u>Binding to pAb</u>                                | <u>Binding to mAb 166</u>                     |
| 10   | 1-294 (full-length)  | yes  | yes   |
|  | (C-term truncates)<br>1-46<br>1-76<br>1-134<br>1-172<br>1-75 + 173-294                                     | yes<br>yes<br>yes<br>yes<br>yes                      | no<br>no<br>no<br>no<br>no                    |
| 15   | (N-term truncates)<br>139-294<br>148-294<br>159-294<br>164-294<br>194-294<br>261-294<br>269-294<br>278-294 | yes<br>yes<br>yes<br>yes<br>yes<br>yes<br>yes<br>yes | yes<br>no<br>no<br>no<br>no<br>no<br>no<br>no |
| 20   |  |  |   |
| 25   |  |  |   |

|    | Amino Acids          | Binding to pAb | Binding to mAb 166 |
|----|----------------------|----------------|--------------------|
|    | (internal fragments) |                |                    |
| 5  | 139-191              | yes            | no                 |
|    | 139-195              | yes            | no                 |
|    | 139-234              | yes            | no                 |
|    | 139-243              | yes            | no                 |
|    | 139-256              | yes            | no                 |
|    | 139-257              | yes            | yes; weak          |
|    | 139-258              | yes            | yes                |
| 10 | 139-259              | yes            | yes                |
|    | 139-260              | yes            | yes                |
|    | 139-261              | yes            | yes                |
|    | 139-262              | yes            | yes                |
|    | 139-263              | yes            | yes                |
| 15 | 139-264              | yes            | yes                |
|    | 139-265              | yes            | yes                |
|    | 139-266              | yes            | yes                |
|    | 139-274              | yes            | yes                |
|    | 139-281              | yes            | yes                |
| 20 | 140-266              | yes            | yes                |
|    | 141-266              | yes            | yes                |
|    | 142-266              | yes            | yes                |
|    | 143-266              | yes            | yes                |
|    | 144-266              | yes            | yes                |
| 25 | 145-266              | yes            | no                 |
|    | 146-266              | yes            | no                 |
|    | 147-266              | yes            | no                 |
|    | 148-170              | no*            | no                 |
|    | 148-202              | yes            | no                 |
| 30 | 159-202              | yes            | no                 |
|    | 159-209              | yes            | no                 |
|    | 159-216              | yes            | no                 |
|    | 159-226              | yes            | no                 |
|    | 159-234              | yes            | no                 |
| 35 | 164-234              | yes            | no                 |
|    | 164-243              | yes            | no                 |
|    | 164-256              | yes            | no                 |
|    | 164-266              | yes            | no                 |
|    | 164-275              | yes            | no                 |
| 40 | 164-281              | yes            | no                 |
|    | 194-234              | yes            | no                 |
|    | 194-243              | yes            | no                 |
|    | 194-256              | yes            | no                 |
|    | 194-266              | yes            | no                 |
| 45 | 194-275              | yes            | no                 |
|    | 194-281              | yes            | no                 |

|    | <u>Amino Acids</u>  | <u>Binding to pAb</u> | <u>Binding to mAb 166</u> |
|----|---|-----------------------|---------------------------|
| 5  | 141-258   | yes                   | yes; weak                 |
|    | 142-258   | NT**                  | yes                       |
|    | 143-258   | yes                   | yes                       |
|    | 144-258   | yes                   | yes                       |
|    | 141-257   | yes                   | yes                       |
|    | 142-257   | yes                   | yes                       |
|    | 143-257   | yes                   | yes                       |
|    | 144-257   | yes; weak             | yes; weak                 |
| 10 | <u>NOTES:</u>   |                       |                           |
|    | *Truncate 148-170 is the only one that is not recognized by the rabbit polyclonal control antibody. |                       |                           |
|    | **NT; Not Tested due to an insufficient amount of bacterial lysate.                                 |                       |                           |
|    | -As predicted, pGEX-4T-2 vector control lysates were not recognized by either antibody.             |                       |                           |
|    | -The smallest epitope of PcrV recognizable by mAb166 appears to consist of amino acids 144-257.     |                       |                           |

15     6.     Examination of PcrV-Specific Antibody

Methods:

Poly A+ RNA extraction: Hybridoma cell line m166 was cultured in complete Dulbeccos minimum essential medium with 4.5 g/L D-glucose, 10 mM HEPES, 50 µM 2-mercaptoethanol, 3 mM L-glutamine, and 10% heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin sulfate. After the cells reached confluent state in a 75 cm<sup>2</sup> flask, the cells were harvested from centrifuging at 600 rpm for 5 minutes. The pellet of the cells was homogenized in 2 mL of TRIzol reagent (Life Technologies, Gaithersburg, MD), and total RNA (100 µg) was extracted after chloroform fractionation, isopropanol precipitation and 70% ethanol wash. Poly A+ RNA (4 µg) was extracted with oligotex mRNA spin-column (Qiagen, Valencia, CA).

RNA oligo-capping: mRNA (250 ng) was incubated with calf intestinal phosphatase at 50°C for 1 hour to dephosphorylate non-mRNA or truncated mRNA. After the reaction, phenol/chloroform extraction and ethanol precipitation was performed and the dephosphorylated RNA was incubated with tobacco acid pyrophosphatase at 37°C for 1 hour to remove the 5'-cap structure from full-length mRNA. After phenol/chloroform extractions and ethanol precipitation, the synthetic RNA oligo (GeneRacer RNA Oligo, Invitrogen, Carlsbad, CA) was ligated to the decapped RNA with T4 RNA

ligase at 37°C for 1 hour. After phenol/chloroform extraction and ethanol precipitation, the RNA was suspended in 13 µL diethylpyrocarbonate-treated water.

Reverse-transcribing mRNA: The RNA-oligo ligated, full-length mRNA 5 (13 µL) was reverse-transcribed with 54 base pair primer containing a dT tail of 18 nucleotides (GeneRacer Oligo dT, Invitrogen), and Avian myeloblastosis virus reverse transcriptase at 42°C for 1 hour in 20 µL reaction. After the reaction, the sample was diluted 4 times with sterile water.

Amplifying cDNA ends by polymerase chain reaction (PCR): One 10 microliter of the cDNA was used for PCR. The 5' primer from the synthetic RNA oligo sequence (GeneRacer 5' Primer, Invitrogen) and the murine immunoglobulin gamma 2b chain CH1 region specific primer or the murine immunoglobulin kappa chain CL region specific primer were used. The cycling parameters used for the PCR reaction was; 1) 94°C, 2 minutes, 15 2) 94°C, 30 seconds and 72°C, 1 minute, 5 cycle, 3) 94°C, 30 seconds, 70°C, 30 seconds, and 72°C, 1 minutes, 5 cycle, 4) 94°C, 30 seconds, 68°C, 30 seconds, and 72°C, 1 minutes, 20 cycle, 5) 72°C, 10 minutes.

Subcloning and DNA sequencing: PCR products (the murine 20 immunoglobulin gamma 2b chain CH1 region derived fragment and the murine immunoglobulin kappa chain CL region derived fragment) were subcloned into the pCRII vector (TOPO cloning, Invitrogen) and submitted to UCSF Molecular Bioresource Center to analyze the DNA sequence.

SEQ ID NO:1 is the DNA sequence of m166 heavy chain mRNA, SEQ 25 ID NO:2 is the amino acid sequence of the m166 heavy chain (IgG II<sub>b</sub>), SEQ ID NO:3 is the DNA sequence of the m166 light chain mRNA, and SEQ ID NO:4 is the amino acid sequence of the m166 light chain. Figs. 6A, 6B and 7 examine the sequences and supply more detail.

Commercial Implications

One could use the antibody sequence to produce recombinant single chain antibodies that may block PcrV and could also utilize the sequence in gene delivery experiments, where one would deliver eukaryotic vectors that  
5 will then lead to the production of single chain antibodies in animals for prolonged periods. Finally, the sequence could be utilized to humanize the murine monoclonal antibody to produce a product that can be utilized in human patient care. One of skill in the art would look to standard methods such as grafting the antigen binding complementarity determining regions  
10 (CDRs) from variable domains of rodent antibodies on to human variable domains in order to create a humanized antibody.

7. Single Chain Antibody Against PcrV

a. Assembling a single chain antibody:

VH gene and VL gene were multiplied by polymerase chain reaction  
15 (PCR) with specific primers for each gene. Multiplied VH and VL fragments were assembled with a linker by using PCR with primers. The assembled single chain antibody gene (scFv::m166:VH and VL genes with linker) was transferred into the cloning vector pCR4 Topo (Invitrogen, Carlsbad, CA). Then, the coding sequence of scFv::m166 was subcloned into the *E. coli*  
20 expression vector pBAD/gIII (Invitrogen) in LMG194 as the host *E. coli*.

b. Protein induction and purification:

For protein induction, in RM medium containing 0.2% glucose and 100 µg of ampicillin, the transformed *E. coli* was cultured overnight at 37°C in an orbital shaker (200 rpm), and the next day, 5 mL of the cultured *E. coli* was  
25 transferred into 500 mL of the same medium and incubated for 3 hours at room temperature at 100 rpm. After L-arabinose was added at the concentration of 0.004%, the *E. coli* was cultured overnight. The third day, the protein was harvested from the periplasmic space of the *E. coli* by

osmotic shock methods. The solution including osmotic shock derived periplasmic protein was dialyzed overnight against the lysis buffer. During the fourth day, the dialyzed solution was applied onto a nickel-NTA column to purify the hexahistidine-tagged single chain antibody. The eluted solution 5 from the nickel column was dialyzed against phosphate buffered saline overnight. On the fifth day, the dialyzed solution was applied to a centrifuge concentrator to make a higher concentration of scFv:m166.

c. The binding test:

10 The purified single chain antibody (scF::m166) was tested by using an enzyme linked immunosorbent assay against recombinant PcrV and by immunoblot (western blot) against both recombinant PcrV protein and native PcrV of *P. aeruginosa* PA103.

15 The single chain antibody will allow us to humanize the antibody utilizing phage-display techniques and to improve affinity of the antibody using these techniques. The single chain antibody can be utilized as a diagnostic tool (for histology) but would not be utilized as a therapy. However, the gene for the single chain antibody can be utilized in gene therapy, so that animals would produce single-chain antibodies over an interval, which could lead to protection against *P. aeruginosa* infections.

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23. Aidi, Y. and Pabst, M.J., "Removal of endotoxin from protein solutions by phase separation using Triton X-114," J. Immunol. Methods 132:191-195 (1990).

## CLAIMS

We claim:

1. A method of inhibiting *Pseudomonas aeruginosa* infection comprising inoculating a patient with an effective amount of PcrV antigen.
2. The method of claim 1 wherein the PcrV antigen is a fragment of the PcrV protein, said fragment capable of inducing an immune response specific to the V antigen.
3. The method of claim 1 wherein the patient is inoculated with a gene vaccine comprising DNA encoding PcrV.
4. The method of claim 3 wherein the DNA encodes a fragment of the PcrV protein, said fragment capable of inducing an immune response specific to the PcrV antigen.
5. The method of claim 1 wherein the patient is a human patient.
6. A method of diagnosing *Pseudomonas aeruginosa* infection comprising the step of exposing a patient's sample to a nucleotide probe, wherein the probe hybridizes specifically to a PcrV-encoding nucleic acid and not to other nucleic acids.
7. The method of claim 6 wherein the patient is a human patient.
8. A method of diagnosing a *Pseudomonas aeruginosa* infection comprising the steps of

- a) exposing a patient's sample to nucleotide primers designed to amplify the *pcrV* gene,
- b) performing a polymerase chain reaction, wherein the *pcrV* gene is amplified if present in the sample, and
- c) correlating *Pseudomonas aeruginosa* infection with the presence of an amplified product.

9. The method of claim 8 wherein the patient is a human patient.

10. A method of diagnosing a *Pseudomonas aeruginosa* infection, comprising the steps of:

- a) exposing the patient sample to a PcrV antigen, and
- b) correlating *Pseudomonas aeruginosa* infection with the presence of a PcrV-specific antibody/antigen complex.

11. A method of inhibiting *Pseudomonas aeruginosa* infection comprising inoculating a patient with an effective amount of a gene vaccine, wherein the gene vaccine encodes PcrV antigen.

12. The method of claim 11 wherein the gene vaccine encodes the entire PcrV protein.

13. The method of claim 11 wherein the gene vaccine encodes a fragment of the PCR PcrV protein, wherein the fragment is capable of inducing an immune response specific to the PcrV antigen.

14. The method of claim 11 wherein the patient is a human patient.

15. A method of treating or preventing *Pseudomonas aeruginosa* infection comprising the steps of obtaining a humanized or human PcrV antibody or antibody fragment, and administering the antibody systemically, wherein the antibody inhibits or prevents *Pseudomonas aeruginosa* infection.
16. A method of treating or preventing *Pseudomonas aeruginosa* infection comprising the steps of obtaining a humanized or human PcrV antibody or antibody fragment and administering the antibody to the lungs as a therapeutic agent.
17. A method of treating a *Pseudomonas aeruginosa* infection comprising the step of inoculating a *Pseudomonas aeruginosa*-infected patient with an effective amount of PcrV antigen.
18. An antibody specific for the PcrV antigen.
19. The antibody of claim 18, wherein the antibody is a monoclonal.
20. The antibody of claim 19, wherein the antibody is mab 166.
21. An anti-PcrV monoclonal antibody or fragment thereof that recognizes an epitope that includes amino acid residues 144 and 257 in the PcrV polypeptide amino acid sequence.
22. An anti-PcrV monoclonal antibody or fragment thereof that recognizes an epitope that includes amino acid residues 144 through 257 in the PcrV polypeptide amino acid sequence.

23. A monoclonal antibody or fragment thereof comprising the CDR's of the light chain polypeptide amino acid sequence shown in Fig. 7.
24. A monoclonal antibody or fragment thereof comprising the CDR's of the heavy chain polypeptide amino acid sequence shown in Fig. 6B.
25. A monoclonal antibody or fragment thereof comprising the CDR's of the light chain polypeptide amino acid sequence shown in Fig. 7 and the CDR's of the heavy chain polypeptide amino acid sequence shown in Fig. 6B.
26. The monoclonal antibody or fragment of any of claims 23 and 25 that further comprises the FR regions of the light chain polypeptide amino acid sequence shown in Fig. 7.
27. The monoclonal antibody or fragment of any of claims 24 and 25 that further comprises the FR regions of the heavy chain polypeptide amino acid sequence shown in Fig. 6B.
28. The monoclonal antibody or fragment of claim 25 that further comprises the FR regions of the heavy chain polypeptide amino acid sequence shown in Fig. 6B and the FR regions of the light chain polypeptide amino acid sequence shown in Fig. 7.
29. The antibody or fragment of any of claims 21-28 that is humanized.
30. The antibody or fragment of any of claims 21-28 that is human.

31. A pharmaceutical composition comprising the antibody or fragment of any of claims 21-30 and a pharmaceutically acceptable carrier.

32. A pharmaceutical composition comprising the antibody or fragment of any of claims 21-30 in an amount effective for treating or preventing Pseudomonas infection in a patient.

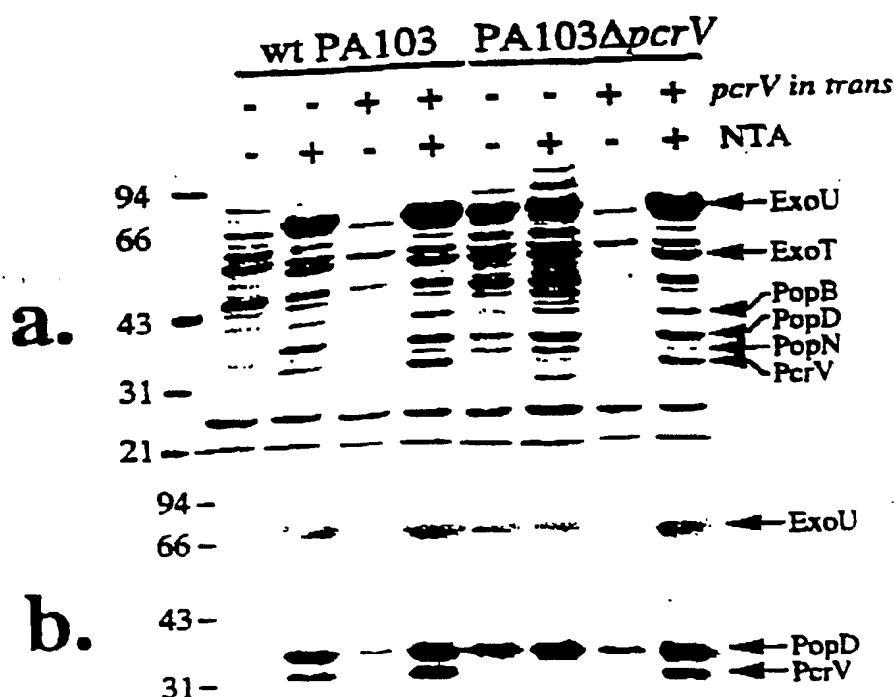
33. A pharmaceutical composition comprising the antibody or fragment of any of claims 21-30 in an amount effective for reducing the pathogenicity of Pseudomonas in a patient.

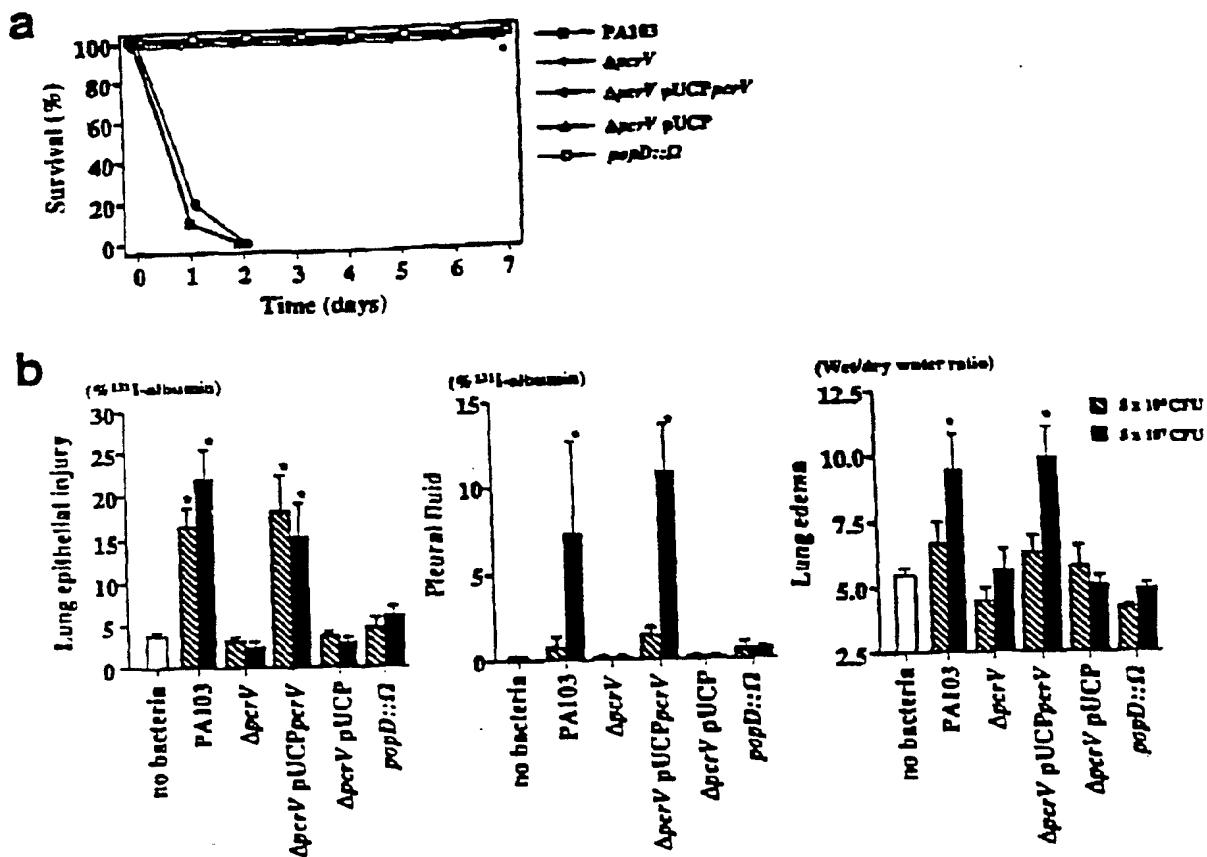
34. A method for treating or preventing Pseudomonas infection in a patient comprising administering to the patient an effective amount of the composition of claim 31 or 32.

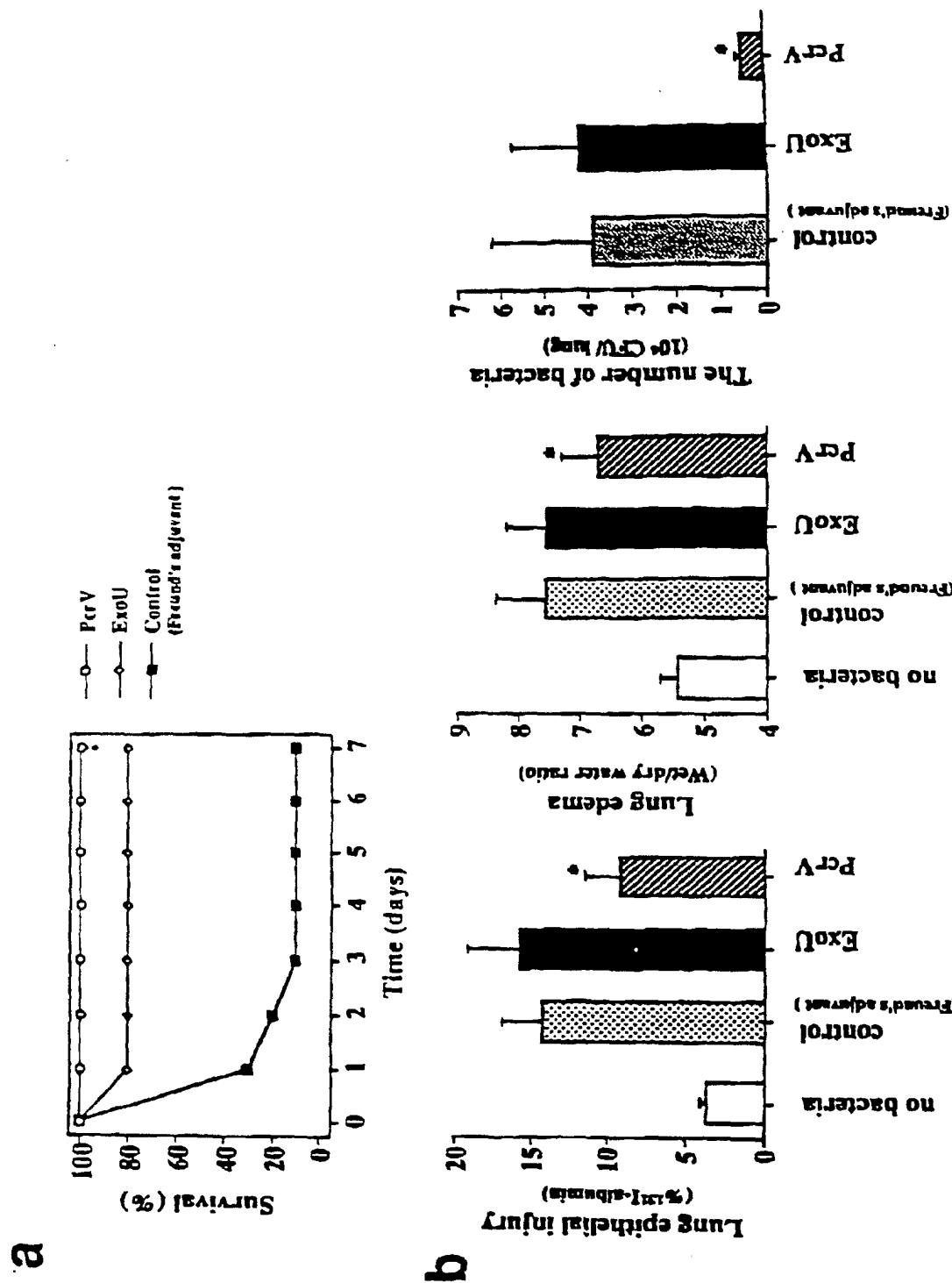
35. A method for reducing the pathogenicity of Pseudomonas in a patient comprising administering to the patient an effective amount of the composition of claim 31 or 33.

36. A method for modulating the cytotoxicity of Pseudomonas to a human cell comprising contacting said Pseudomonas with the antibody or fragment of any of claims 21-30 in the presence of the human cell.

37. A nucleic acid encoding the antibody or fragment of any of claims 21-30.

**FIG. 1**

**FIG. 2**



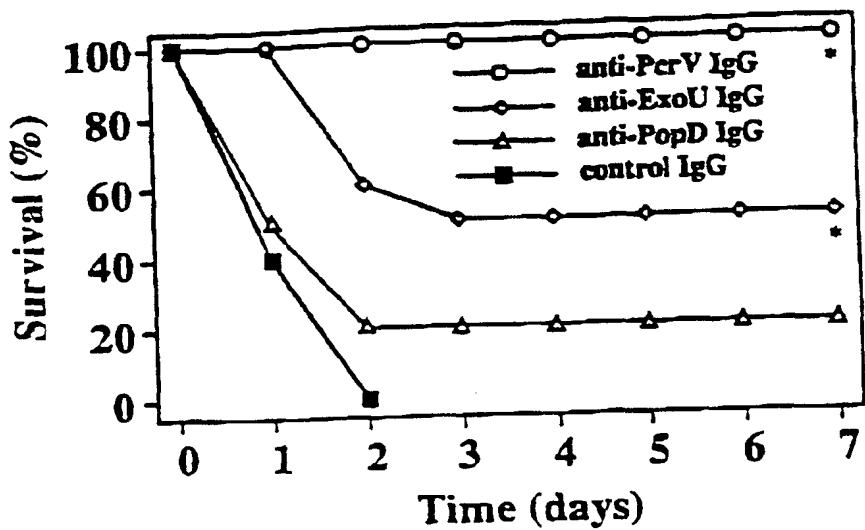
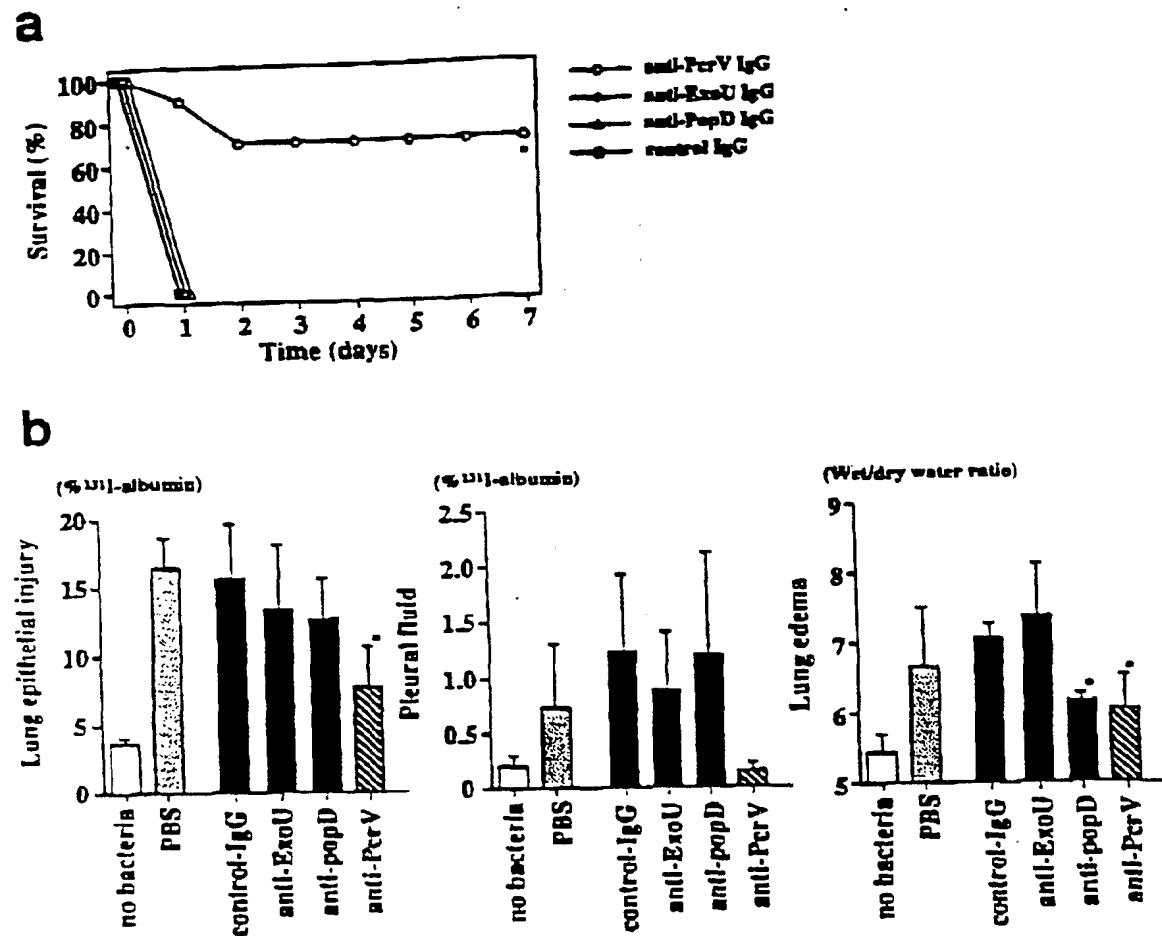


FIG. 4

**FIG. 5**

## m166 heavy chain

### 1. m166 heavy chain (IgG2b) complete mRNA sequence:

(From the transcriptional start point to the polyA-tail)

```
CCATCCTCTT CTCATAGAGC CTCCATCAGA GCATGGCTGT CITGGGGCTG
CTCTCTGCC TGGTGACATT CCCAAGCTGT GTCCTATCCC AGGTGCAGCT
GAAGCAGTCA GGACCTGGCC TAGTGCAGCC CTCACAGAGC CTGTCATCA
CCTGCACAGT CTCTGGTTTC TCATTAACTA GCTATGGTGT ACACCTGGTT
CGTCAGTCCTC CAGGAAAGGG TCTGGAGTGG CTGGGAGTGA TATGGAGTGG
TGGAGACACA GACTATAATG CAGCTTTCAT ATCCAGACTG AGCATCAGCA
AGGACAATTG CAAGAGCCAA CTCTCTTTA AAATGAACAG TCTGCGAGCT
ACTGACACAG CCATATATTA CTGTCAGAGA AATAGAGGG ATATTTACTA
TGATTTCACT TATGCCATGG ACTACTGGGG TCAAGGAACC TCAGTCACCG
TCCTCTCAGC CAAAACAAACA CCCCCATCAG TCTATCCACT GGCCCCCTGGG
TGTGGAGATA CAACTGGTTC CTCCGTGACT CTGGGATGCC TGGTCAAGGG
CTACTTCCCT GAGTCAGTGA CTGTGACTTG GAACTCTGGA TCCCTCTCCA
GCACTGTGCA CACCTTCCCA GCTCTCTGC AGTCTGGACT CTACACTATG
AGCAGCTCAG TGACTGTCCC CTCCAGCACC TGGCCAAGTC AGACCGTCAC
CTGCAAGGGT GCTCACCCAG CCAGCAGCAC CACGGTGGAC AAAAAACTTG
AGCCCCAGGG GCCCATTTCA ACAATCAACC CCTGTCCTCC ATGCAAGGAG
TGTCAAAAT GCCCCAGCTCC TAACCTCGAG GGTGGACCAT CCGTCTTCAT
CTTCCCTCCA AATATCAAGG ATGTAACAT GATCTCCCTG ACACCCAAAGG
TCACGTGTGT GGTGGTGGAT GTGAGCGAGG ATGACCCAGA CGTCCAGATC
AGCTGGTTTG TGAACAAACGT GGAAGTACAC ACAGCTCAGA CACAAACCCA
TAGAGAGGGAT TACAACAGTA CTATCCGGT GGTCAAGCACC CTCCCCATCC
AGCACCAGGA CTGGATGAGT GSCAAGGGT TCAAATGCA GGTCAACAAC
AAAGACCTCC CATCACCCAT CGAGAGAACCC ATCTCAAAAA TTAAAGGGCT
AGTCAGAGCT CCACAAGTAT ACATCTTGCC GCCACCAGCA GAGCACTTGT
CCAGGAAAGA TGTCACTTC ACTTGCCTGG TCGTGGGCTT CAAACCTGG
GACATCAGTG TGGAGTGGAC CAGCAATGGG CATAAGAGGG AGAAACTACAA
GGACACCGCA CCAGTCTGG ACTCTGACGG TTCTTACTTC ATATATAGCA
AGCTCAATAT GAAAACAAAGC AAGTGGGAGA AAACAGATTG CTTCTCATGC
AACGTGAGAC AGCAGGGTCT GAAAATTAC TACCTGAAGA AGACCATCTC
CCGGTCTCCG CGTAARTGAG CTCAGGCACCC ACAAAAGCTCT CAGGTCCCTAA
GAGACACTGG CACCCATATC CATGCATCCC TTGTATAAT AAAGCATCCA
GCAAAGCTCG GTACCATGTA AAAAAAAAAA AAAAAAAA
```

**FIG. 6A**

**2. m166 heavy chain (IgG2b) complete amino acid sequence:**

(From the start codon to the stop codon)

```
-----  
MAVLGLLFCL VTFPSCVLSQ VQLKQSGPGL VOPSQSLISIT CTVSGFSLTS  
YGVHWRQSP GKGLEWLGV1 WSGGDTDYNA AFISRLSISK DNSKSQFFK  
MNSLRATDTA IYYCARNRGD IYYDFTYAMD YWQGTSVTIV SSAKTTPPSV  
YPLAFCGCGDT TGSSVTLGCL VKGYFPESTV VTIWNSSLSS SVHTFPALLQ  
SGLYTMSSSV TVPSSTWPSSQ TVTCVAHFA SSTTVDKKLE PSGPISTINP  
CPPCKECHKC PAPNLEGGPS VFIFPPNIKD VLMISLTPKV TCVVVDVSED  
DPDVQISWVF NNVEVHTAQQT QTHREDYNST IRVVSILPIQ HQDWMSGKEF  
KCKVNNKDLR SPIERTISKI KGLVRAPQVY ILPPPAEQLS RKDVSLLTCLV  
VGFNPDDISV EWTSNGKTEE NYKDTAPVLD SDGSYFIYSK LNMMKTSKWEK  
TDSFSCNVRH EGLKNYYLKK TISRSPGK [STOP]  
-----
```

[Sig-pep] MAVLGLLFCLVTFPSCVLS

[VH-region]

FR1: QVQLKQSGPGLVQPSQSLISITCTVSGFSLT  
CDR1: SYGVH  
FR2: WVRQSPGKGLEWLG  
CDR2: VIWSGGDTDYNAAFIS  
FR3: RLSISKDNSKSQFFKMNSLRATDTAIYYCAR  
CDR3: NRGDIYYDFTYAMDY  
FR4: WGQGTSVTVSS

[CH-region]

CH:  
AKTTPPSVYF LAPGCGDTTG SSVTLGCLVK GYFPESVTVT WNSGSLSSSV  
HTFPALLQSG LYTMSSSVTV PSSTWPSSQTV TCSVAHPASS TTVDKKEPS  
GPISTINPCP PCKECHKCPSA PNLEGGPSVF IFPPNIKDVL MISLTPKVTC  
VVVDVSEDDP DVQISWVNN VEVHTAQQTQ THREDYNSTIR VVSTLPQHQ  
DWMSGKEFKC KVNNKDLRSP IERTISKIKG LVRAPQVYIL PPPAEQLSRRK  
DVSLTCLVVG FNPGDISVEW TSNGKTEENY KDTAPVLDSD GSYFIYSKLN  
MKTSKWEKTD SFSCNVRHEG LKNYYLKKTI SRSPGK[STOP]

**FIG. 6B**

## m166 light chain

### 1. m166 light chain (k) complete mRNA sequence:

(From the transcriptional start point to the polyA tail)

```
-----  
ACACCCCTTG CTGGAGTCAG AATCACACTG ATCACACACA GTCATGAGTG  
TGCTCACTCA GGTCCTGGCG TTGCTGCTGC TGTGGCTTAC AGGTGCCAGA  
TGTGACATCC AGATGACTCA GTCTCCAGCC TCCCTATCTG CATCTGTGGG  
AGAAACTGTC ACCATCACAT GTCGAGCAAG TGGGAATATT CAAAATTATT  
TAGCATGGTA TCAGCAGACA CAGGGAAAAT CCTCCAGCT CCTGGTCTAT  
TCTGCAAAA CCTTAGCAGA TGGTGTGCCA TCAAGGTTCA GTGGCAGTGG  
ATCAGGAACA CAATATTCTC TCAAGATCAA CAGCCIGCAG CCTGAAGATT  
TTGGGAGTTA TTACTGTCAA CATTITGGA GTACTCCGTA CACGTTCGGA  
GGGGGGACCA AGCTGAAAT AAAACGGGCT GATGCTGAC CAACTGTATC  
CATCTTCCA CCATCAGTG AGCAGTTAAC ATCTGGAGGT GCCTCAGTCG  
TGTGCTTCTT GAACAACCT TACCCCAAAG ACATCAATGT CAAGTGGAAAG  
ATTGATGGCA GTGAAACGACA AAATGGCGTC CTGAAACAGTT GGACTGATCA  
GGACAGCAA GACAGCACCT ACAGCATGAG CAGCACCCCTC ACGTTGACCA  
AGGACGAGTA TGAACGACAT AACAGCTATA CCTGTGAGGC CACTCACAAG  
ACATCAACTT CACCCATTGT CAAGAGCTTC AACAGGAATG AGTGGTAGAG  
ACAAAGGTCG TGAGACGCCA CCACCAAGCTC CCCAGCTCCA TCCTATCTC  
CCTTCTAAGG TCTTGGAGGC TTCCCCACAA GCGACCTACC ACTGGTGCAG  
TGCTCCAAAC CTCCCTCCC CCTCCTCTC CTCCCTCTCC TTTCCTTGG  
CTTTATCAT GCTAATATTG GCAGAAAATA TTCAATAAG TGAGTCTTIG  
AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA  
-----
```

### 2. m166 light chain (k) amino acid complete sequence:

(From the start codon to the stop codon)

```
-----  
MSVLTVLALLLWLWLTGARC DIQMTQSPAS LSASVGETVT ITCRASGNIQ  
NYLAWYQQTQ GKSPQLLVYS AKTLADGVPS RFSGSGSGTQ YSLKINSLQP  
EDFGSYYCQH FWSTPYTFGG GTKLEIKRAD AAPTVSIFPP SSEQLTSGGA  
SVVCFLNNFY PKDINVWKI DGSERQNGVL NSWTQDQDSKD STYSMSSTLT  
LTKDEYERHN SYTCEATHKT STSPIVKSFN RNEC [STOP]  
-----
```

[Sig-pep] MSVLTVLALLLWLWLTGARC

[VL-region]

FR1: DIQMTQSPASLSASVGETVTITC

CDR1: RASGNIQNYLA

FR2: WYQQTQGKSPQLLVY

CDR2: SAKTLAD

FR3: GVPNSRFSGSGSGTQYSLKINSLQPEDFGSYYC

CDR3: QHFWSTPYT

FR4: FGGGTKLEIKR

[CL-region]

CL: ADAAPTVSIFPPSSEOLTSGGASVVCFLNNFYPKDINVWKIDGSERQNGVL

NSWTQDQDSKDSTYSMSSTLT LTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC [STOP]

**FIG. 7**

## Synthetic recombinant single chain antibody (scFv-m166)

### 1. DNA sequence:

```
ATGAAAAAAAC TGCTGTTCGC GATTCCGCTG GTGGTGCCTG TCTATAGCCA
TAGCACCATG GAGCTCGAGC GGCAGGTGCA GCTGAAGCAG TCAGGACCTG
GCCTAGTGC GGCCTCACAG AGCCTGTC CA TCACCTGCAC AGTCTCTGGT
TTCTCATTA CTAGCTATGG TGATACACTGG GTTCTGCACT CTCAGGAAA
GGGTCTGGAG TGGCTGGGAG TGATATGGAG TGGTGGAGAC ACAGACTATA
ATGCAGCTTT CATATCCAGA CTGAGCATCA GCAAGGACA ITCCAAGAGC
CAAECTCTCTT TAAATAGAA CAGTCTGGG GCTACTGACA CAGCCATATA
TTACTGTGCC AGAAATAGAG GGGATATTCA CTATGATTTC ACTTATGCCA
TGGACTACTG GGGTCAGGAA ACCTCAGTCA CCGTCTCCTC AGGTGGAGGC
GGCTCAGGG GAGGTGGCTC TGGCGGTGGC GGATCGGACA TCCAGATGAC
TCAGTCTCCA GCCTCCCTAT CTGCATCTGT GGGAGAAACT GTCACCATCA
CATGTCCAGC ARGTGGGAAT ATTCAAAATT ATTTAGCATG GTATCAGCAG
ACACACGGAA AATCTCCCTCA GCTCCCTGGTC TATTCTGCAAA AACCTTAC
AGATGGTGTG CCATCAAGGT TCAGTGGAG TGGCATCAGGA ACACAATATT
CTCTCAAGAT CAACAGCCTG CAGCCTGAAG ATTTTGGGAG TTATTACTGT
CAACATTTTG GGAGTACTCC GTACACGTT GGAGGGGGGA CCAAGCTGGA
AATAAAACGG GCTCTAGAAC AAAACTCAT CTCAGAAGAG GATCTGAATA
GCCCGCTCGA CCATCATCAT CATCATCATT GA
```

### 2. Amino acid sequence:

```
MKKLLFAIPL VVPFYSHSTM ELERQVQLKQ SGPGIVRPSQ SLSITCTIVSG
FSLTSYGVHW VRQSPGKGLE WLGVIVSGGD TDYNAFISR LSISKDNNSKS
QLFFKMNLSLR ATDTAIYYCA RNRGDIYYDF TYAMDYWGQG TSIVSSGGG
GSGGGGSGGG GSIDIQMTQSP ASLSASVGET VTITCRASNQ IONYLAWYQQ
TQGKSPQLLV YSAKTLADGV PSRFSGSGSG TQYSLKINSQ QPEDFGSYYC
QHFWSTPYTF GGGTKLEIKR ALEQKLISEE DLNSAVDHHH HHH [STOP]
```

[Gene III signal sequence] MKKLLFAIPLVVFYSHS  
 [Joint-1] TMELER

[m166 heavy chain]  
 QVQLKQSGPG LVRPSQSLSI TCTVSGFSLT SYGVHWRQD PGKGLEWLGV
 IWSGGDTDYN AAFISRLSIS KONSKSQLFF KMNSLRATDT AIYYCARNRG
 DIYYDFTYAM DYWGQGTSVT VSS

[scFv-linker] GGGGSGGGGGGGGG

[m166 light chain]  
 DIQMTQSPAS LSASVGETVT ITCRASNQ NYLAWYQQTQ GKSPQLLVYS
 AKTLADGVPS RFSGSGSGTQ YSLKINSLQP EDFGSYYCQH FWSTPYTFGG
 GTKLEIKR

|                     |               |
|---------------------|---------------|
| [Joint-2]           | AL            |
| [Myc epitope]       | EQKLISEEDL    |
| [Joint-3]           | NSAVD         |
| [Hexahistidine tag] | HHHHHH [STOP] |

**FIG. 8**

## SEQUENCE LISTING

<110> Frank, Dara W.  
Wiener-Kronish, Jeannine  
Yahr, Timothy L.  
Sawa, Teiji  
Fritz, Robert B.

<120> Method of and compositions for immunization with the  
pseudomonas V antigen

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Phe Cys Leu Val Thr Phe Pro Ser Cys Val Leu Ser Gln Val Gln Leu  
10 15 20

aag cag tca gga cct ggc cta gtg cag ccc tca cag agc ctg tcc atc 149  
Lys Gln Ser Gly Pro Gly Leu Val Gln Pro Ser Gln Ser Leu Ser Ile

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acc tgc aca gtc tct ggt ttc tca tta act agc tat ggt gta cac tgg 197  
 Thr Cys Thr Val Ser Gly Phe Ser Leu Thr Ser Tyr Gly Val His Trp  
 40                45                50                55

gtt cgt cag tct cca gga aag ggt ctg gag tgg ctg gga gtg ata tgg 245  
 Val Arg Gln Ser Pro Gly Lys Gly Leu Glu Trp Leu Gly Val Ile Trp  
 60                65                70

agt ggt gga gac aca gac tat aat gca gct ttc ata tcc aga ctg agc 293  
 Ser Gly Gly Asp Thr Asp Tyr Asn Ala Ala Phe Ile Ser Arg Leu Ser  
 75                80                85

atc agc aag gac aat tcc aag agc caa ctc ttc ttt aaa atg aac agt 341  
 Ile Ser Lys Asp Asn Ser Lys Ser Gln Leu Phe Phe Lys Met Asn Ser  
 90                95                100

ctg cga gct act gac aca gcc ata tat tac tgt gcc aga aat aga ggg 389  
 Leu Arg Ala Thr Asp Thr Ala Ile Tyr Tyr Cys Ala Arg Asn Arg Gly  
 105                110                115

gat att tac tat gat ttc act tat gcc atg gac tac tgg ggt caa gga 437  
 Asp Ile Tyr Tyr Asp Phe Thr Tyr Ala Met Asp Tyr Trp Gly Gln Gly  
 120                125                130                135

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 Asn Ser Gly Ser Leu Ser Ser Val His Thr Phe Pro Ala Leu Leu  
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 220 225 230

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 Ser Thr Thr Val Asp Lys Lys Leu Glu Pro Ser Gly Pro Ile Ser Thr  
 235 240 245

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 Ile Asn Pro Cys Pro Pro Cys Lys Glu Cys His Lys Cys Pro Ala Pro  
 250 255 260

aac ctc gag ggt gga cca tcc gtc ttc atc ttc cct cca aat atc aag 869  
 Asn Leu Glu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Asn Ile Lys  
 265 270 275

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 280 285 290 295

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 Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe Val Asn  
 300 305 310

aac gtg gaa gta cac aca gct cag aca caa acc cat aga gag gat tac 1013  
 Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu Asp Tyr  
 315 320 325

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 Asn Ser Thr Ile Arg Val Val Ser Thr Leu Pro Ile Gln His Gln Asp  
 330 335 340

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 Pro Ser Pro Ile Glu Arg Thr Ile Ser Lys Ile Lys Gly Leu Val Arg  
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 Ala Pro Gln Val Tyr Ile Leu Pro Pro Ala Glu Gln Leu Ser Arg  
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 Lys Asp Val Ser Leu Thr Cys Leu Val Val Gly Phe Asn Pro Gly Asp

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 Asp Thr Ala Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Ile Tyr Ser  
 425                  430                  435

aag ctc aat atg aaa aca agc aag tgg gag aaa aca gat tcc ttc tca 1397  
 Lys Leu Asn Met Lys Thr Ser Lys Trp Glu Lys Thr Asp Ser Phe Ser  
 440                  445                  450                  455

tgc aac gtg aga cac gag ggt ctg aaa aat tac tac ctg aag aag acc 1445  
 Cys Asn Val Arg His Glu Gly Leu Lys Asn Tyr Tyr Leu Lys Lys Thr  
 460                  465                  470

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 Ile Ser Arg Ser Pro Gly Lys  
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 Met Ala Val Leu Gly Leu Leu Phe Cys Leu Val Thr Phe Pro Ser Cys  
 1                  5                  10                  15

Val Leu Ser Gln Val Gln Leu Lys Gln Ser Gly Pro Gly Leu Val Gln  
 20                  25                  30

Pro Ser Gln Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser Leu  
 35                  40                  45

Thr Ser Tyr Gly Val His Trp Val Arg Gln Ser Pro Gly Lys Gly Leu  
 50                  55                  60

Glu Trp Leu Gly Val Ile Trp Ser Gly Gly Asp Thr Asp Tyr Asn Ala

65            70            75            80  
Ala Phe Ile Ser Arg Leu Ser Ile Ser Lys Asp Asn Ser Lys Ser Gln  
85            90            95  
Leu Phe Phe Lys Met Asn Ser Leu Arg Ala Thr Asp Thr Ala Ile Tyr  
100            105            110  
Tyr Cys Ala Arg Asn Arg Gly Asp Ile Tyr Tyr Asp Phe Thr Tyr Ala  
115            120            125  
Met Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser Ala Lys  
130            135            140  
Thr Thr Pro Pro Ser Val Tyr Pro Leu Ala Pro Gly Cys Gly Asp Thr  
145            150            155            160  
Thr Gly Ser Ser Val Thr Leu Gly Cys Leu Val Lys Gly Tyr Phe Pro  
165            170            175  
Glu Ser Val Thr Val Thr Trp Asn Ser Gly Ser Leu Ser Ser Ser Val  
180            185            190  
His Thr Phe Pro Ala Leu Leu Gln Ser Gly Leu Tyr Thr Met Ser Ser  
195            200            205  
Ser Val Thr Val Pro Ser Ser Thr Trp Pro Ser Gln Thr Val Thr Cys  
210            215            220  
Ser Val Ala His Pro Ala Ser Ser Thr Thr Val Asp Lys Lys Leu Glu  
225            230            235            240  
Pro Ser Gly Pro Ile Ser Thr Ile Asn Pro Cys Pro Pro Cys Lys Glu  
245            250            255  
Cys His Lys Cys Pro Ala Pro Asn Leu Glu Gly Gly Pro Ser Val Phe  
260            265            270  
Ile Phe Pro Pro Asn Ile Lys Asp Val Leu Met Ile Ser Leu Thr Pro  
275            280            285  
Lys Val Thr Cys Val Val Val Asp Val Ser Glu Asp Asp Pro Asp Val  
290            295            300  
Gln Ile Ser Trp Phe Val Asn Asn Val Glu Val His Thr Ala Gln Thr  
305            310            315            320

Gln Thr His Arg Glu Asp Tyr Asn Ser Thr Ile Arg Val Val Ser Thr  
325            330            335

Leu Pro Ile Gln His Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys  
340            345            350

Lys Val Asn Asn Lys Asp Leu Pro Ser Pro Ile Glu Arg Thr Ile Ser  
355            360            365

Lys Ile Lys Gly Leu Val Arg Ala Pro Gln Val Tyr Ile Leu Pro Pro  
370            375            380

Pro Ala Glu Gln Leu Ser Arg Lys Asp Val Ser Leu Thr Cys Leu Val  
385            390            395            400

Val Gly Phe Asn Pro Gly Asp Ile Ser Val Glu Trp Thr Ser Asn Gly  
405            410            415

His Thr Glu Glu Asn Tyr Lys Asp Thr Ala Pro Val Leu Asp Ser Asp  
420            425            430

Gly Ser Tyr Phe Ile Tyr Ser Lys Leu Asn Met Lys Thr Ser Lys Trp  
435            440            445

Glu Lys Thr Asp Ser Phe Ser Cys Asn Val Arg His Glu Gly Leu Lys  
450            455            460

Asn Tyr Tyr Leu Lys Lys Thr Ile Ser Arg Ser Pro Gly Lys  
465            470            475

<210> 3

<211> 979

<212> DNA

<213> mouse

<220>

<221> CDS

<222> (44)..(745)

<220>

<221> sig\_peptide

<222> (44)..(103)

<220>  
<221> V\_region  
<222> (104)..(172)  
<223> FR1

<220>  
<221> V\_region  
<222> (173)..(205)  
<223> CDR1

<220>  
<221> V\_region  
<222> (206)..(250)  
<223> FR2

<220>  
<221> V\_region  
<222> (251)..(271)  
<223> CDR2

<220>  
<221> V\_region  
<222> (272)..(367)  
<223> FR3

<220>  
<221> V\_region  
<222> (368)..(394)  
<223> CDR3

<220>  
<221> V\_region  
<222> (455)..(487)  
<223> FR4

<220>  
<221> C\_region  
<222> (428)..(745)

<400> 3  
acacccttg ctggagtcag aatcacactg atcacacaca gtc atg agt gtg ctc 55  
Met Ser Val Leu  
1

act cag gtc ctg gcg ttg ctg ctg tgg ctt aca ggt gcc aga tgt 103  
Thr Gln Val Leu Ala Leu Leu Leu Trp Leu Thr Gly Ala Arg Cys

|   |    |    |    |  |
|---|----|----|----|--|
| 5 | 10 | 15 | 20 |  |
|---|----|----|----|--|

gac atc cag atg act cag tct cca gcc tcc cta tct gca tct gtg gga 151  
 Asp Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly  
 25                30                35

gaa act gtc acc atc aca tgt cga gca agt ggg aat att caa aat tat 199  
 Glu Thr Val Thr Ile Thr Cys Arg Ala Ser Gly Asn Ile Gln Asn Tyr  
 40                45                50

tta gca tgg tat cag cag aca cag gga aaa tct cct cag ctc ctg gtc 247  
 Leu Ala Trp Tyr Gln Gln Thr Gln Gly Lys Ser Pro Gln Leu Leu Val  
 55                60                65

tat tct gca aaa acc tta gca gat ggt gtg cca tca agg ttc agt ggc 295  
 Tyr Ser Ala Lys Thr Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly  
 70                75                80

agt gga tca gga aca caa tat tct ctc aag atc aac agc ctg cag cct 343  
 Ser Gly Ser Gly Thr Gln Tyr Ser Leu Lys Ile Asn Ser Leu Gln Pro  
 85                90                95                100

gaa gat ttt ggg agt tat tac tgt caa cat ttt tgg agt act ccg tac 391  
 Glu Asp Phe Gly Ser Tyr Tyr Cys Gln His Phe Trp Ser Thr Pro Tyr  
 105                110                115

acg ttc gga ggg ggg acc aag ctg gaa ata aaa cgg gct gat gct gca 439  
 Thr Phe Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Asp Ala Ala  
 120                125                130

cca act gta tcc atc ttc cca cca tcc agt gag cag tta aca tct gga 487  
 Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln Leu Thr Ser Gly  
 135                140                145

ggt gcc tca gtc gtg tgc ttc ttg aac aac ttc tac ccc aaa gac atc 535  
 Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr Pro Lys Asp Ile  
 150                155                160

aat gtc aag tgg aag att gat ggc agt gaa cga caa aat ggc gtc ctg 583  
 Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln Asn Gly Val Leu  
 165                170                175                180

aac agt tgg act gat cag gac aaa gac agc acc tac agc atg agc 631  
 Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr Tyr Ser Met Ser  
 185                190                195

agc acc ctc acg ttg acc aag gac gag tat gaa cga cat aac agc tat 679  
 Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg His Asn Ser Tyr  
 200 205 210

acc tgt gag gcc act cac aag aca tca act tca ccc att gtc aag agc 727  
 Thr Cys Glu Ala Thr His Lys Thr Ser Thr Pro Ile Val Lys Ser  
 215 220 225

ttc aac agg aat gag tgt tagagacaaa ggtcctgaga cgccaccacc 775  
 Phe Asn Arg Asn Glu Cys  
 230

agctccccag ctccatccta tcttcccttc taaggcttg gaggctccc cacaagcgac 835

ctaccactgt tgccgtgctc caaacctctt ccccacccctt ctttcctctt cttcccttc 895

cgtggcttt atcatgctaa tattgcaga aaatattcaa taaagttagt ctttgcaaaa 955

aaaaaaaaaaaa aaaaaaaaaaaa aaaa 979

<210> 4  
 <211> 234  
 <212> PRT  
 <213> mouse

<400> 4  
 Met Ser Val Leu Thr Gln Val Leu Ala Leu Leu Leu Trp Leu Thr  
 1 5 10 15

Gly Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser  
 20 25 30

Ala Ser Val Gly Glu Thr Val Thr Ile Thr Cys Arg Ala Ser Gly Asn  
 35 40 45

Ile Gln Asn Tyr Leu Ala Trp Tyr Gln Gln Thr Gln Gly Lys Ser Pro  
 50 55 60

Gln Leu Leu Val Tyr Ser Ala Lys Thr Leu Ala Asp Gly Val Pro Ser  
 65 70 75 80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln Tyr Ser Leu Lys Ile Asn  
 85 90 95

Ser Leu Gln Pro Glu Asp Phe Gly Ser Tyr Tyr Cys Gln His Phe Trp

100                105                110

Ser Thr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg  
115                120                125

Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln  
130                135                140

Leu Thr Ser Gly Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr  
145                150                155                160

Pro Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln  
165                170                175

Asn Gly Val Leu Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr  
180                185                190

Tyr Ser Met Ser Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg  
195                200                205

His Asn Ser Tyr Thr Cys Glu Ala Thr His Lys Thr Ser Thr Ser Pro  
210                215                220

Ile Val Lys Ser Phe Asn Arg Asn Glu Cys  
225                230

<210> 5  
<211> 882  
<212> DNA  
<213> Artificial Sequence

<220>  
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<222> (1)..(54)  
<223> Gene III signal sequence

<220>  
<221> misc\_feature  
<222> (55)..(72)  
<223> Joint-1

<220>  
<221> misc\_feature  
<222> (73)..(441)

<223> m166 heavy chain

<220>  
<221> misc\_feature  
<222> (442)..(486)  
<223> scFv-linker

<220>  
<221> misc\_feature  
<222> (487)..(810)  
<223> m166 light chain

<220>  
<221> misc\_feature  
<222> (811)..(816)  
<223> Joint-2

<220>  
<221> misc\_feature  
<222> (817)..(846)  
<223> Myc epitope

<220>  
<221> misc\_feature  
<222> (847)..(861)  
<223> Joint-3

<220>  
<221> misc\_feature  
<222> (862)..(879)  
<223> Hexahistidine tag

<220>  
<221> CDS  
<222> (1)..(879)

<400> 5  
atg aaa aaa ctg ctg ttc gcg att ccg ctg gtg gtg ccg ttc tat agc 48  
Met Lys Lys Leu Leu Phe Ala Ile Pro Leu Val Val Pro Phe Tyr Ser  
1 5 10 15

cat agc acc atg gag ctc gag cggtt cag ctg aag cag tca gga 96  
His Ser Thr Met Glu Leu Glu Arg Gln Val Gln Leu Lys Gln Ser Gly  
20 25 30

cct ggc cta gtg cgg ccc tca cag agc ctg tcc atc acc tgc aca gtc 144

Pro Gly Leu Val Arg Pro Ser Gln Ser Leu Ser Ile Thr Cys Thr Val  
 35                40                45

tct ggt ttc tca tta act agc tat ggt gta cac tgg gtt cgt cag tct 192  
 Ser Gly Phe Ser Leu Thr Ser Tyr Gly Val His Trp Val Arg Gln Ser  
 50                55                60

cca gga aag ggt ctg gag tgg ctg gga gtg ata tgg agt ggt gga gac 240  
 Pro Gly Lys Gly Leu Glu Trp Leu Gly Val Ile Trp Ser Gly Gly Asp  
 65                70                75                80

aca gac tat aat gca gct ttc ata tcc aga ctg agc atc agc aag gac 288  
 Thr Asp Tyr Asn Ala Ala Phe Ile Ser Arg Leu Ser Ile Ser Lys Asp  
 85                90                95

aat tcc aag agc caa ctc ttc ttt aaa atg aac agt ctg cga gct act 336  
 Asn Ser Lys Ser Gln Leu Phe Phe Lys Met Asn Ser Leu Arg Ala Thr  
 100                105                110

gac aca gcc ata tat tac tgt gcc aga aat aga ggg gat att tac tat 384  
 Asp Thr Ala Ile Tyr Tyr Cys Ala Arg Asn Arg Gly Asp Ile Tyr Tyr  
 115                120                125

gat ttc act tat gcg atg gac tac tgg ggt caa gga acc tca gtc acc 432  
 Asp Phe Thr Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr  
 130                135                140

gtc tcc tca ggt gga ggc ggc tca ggc gga ggt ggc tct ggc ggt ggc 480  
 Val Ser Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly  
 145                150                155                160

gga tcg gac atc cag atg act cag tct cca gcc tcc cta tct gca tct 528  
 Gly Ser Asp Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser  
 165                170                175

gtg gga gaa act gtc acc atc aca tgt cga gca agt ggg aat att caa 576  
 Val Gly Glu Thr Val Thr Ile Thr Cys Arg Ala Ser Gly Asn Ile Gln  
 180                185                190

aat tat tta gca tgg tat cag cag aca cag gga aaa tct cct cag ctc 624  
 Asn Tyr Leu Ala Trp Tyr Gln Gln Thr Gln Gly Lys Ser Pro Gln Leu  
 195                200                205

ctg gtc tat tct gca aaa acc tta gca gat ggt gtg cca tca agg ttc 672  
 Leu Val Tyr Ser Ala Lys Thr Leu Ala Asp Gly Val Pro Ser Arg Phe  
 210                215                220

agt ggc agt gga tca gga aca caa tat tct ctc aag atc aac agc ctg 720  
 Ser Gly Ser Gly Ser Gly Thr Gln Tyr Ser Leu Lys Ile Asn Ser Leu  
 225 230 235 240

cag cct gaa gat ttt ggg agt tat tac tgt caa cat ttt tgg agt act 768  
 Gln Pro Glu Asp Phe Gly Ser Tyr Tyr Cys Gln His Phe Trp Ser Thr  
 245 250 255

ccg tac acg ttc gga ggg ggg acc aag ctg gaa ata aaa cgg gct cta 816  
 Pro Tyr Thr Phe Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Leu  
 260 265 270

gaa caa aaa ctc atc tca gaa gag gat ctg aat agc gcc gtc gac cat 864  
 Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His  
 275 280 285

cat cat cat cat cat tga 882  
 His His His His His  
 290

<210> 6  
 <211> 293  
 <212> PRT  
 <213> Artificial Sequence

<400> 6  
 Met Lys Lys Leu Leu Phe Ala Ile Pro Leu Val Val Pro Phe Tyr Ser  
 1 5 10 15

His Ser Thr Met Glu Leu Glu Arg Gln Val Gln Leu Lys Gln Ser Gly  
 20 25 30

Pro Gly Leu Val Arg Pro Ser Gln Ser Leu Ser Ile Thr Cys Thr Val  
 35 40 45

Ser Gly Phe Ser Leu Thr Ser Tyr Gly Val His Trp Val Arg Gln Ser  
 50 55 60

Pro Gly Lys Gly Leu Glu Trp Leu Gly Val Ile Trp Ser Gly Gly Asp  
 65 70 75 80

Thr Asp Tyr Asn Ala Ala Phe Ile Ser Arg Leu Ser Ile Ser Lys Asp  
 85 90 95

Asn Ser Lys Ser Gln Leu Phe Phe Lys Met Asn Ser Leu Arg Ala Thr  
100 105 110

Asp Thr Ala Ile Tyr Tyr Cys Ala Arg Asn Arg Gly Asp Ile Tyr Tyr  
115 120 125

Asp Phe Thr Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr  
130 135 140

Val Ser Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly  
145 150 155 160

Gly Ser Asp Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser  
165 170 175

Val Gly Glu Thr Val Thr Ile Thr Cys Arg Ala Ser Gly Asn Ile Gln  
180 185 190

Asn Tyr Leu Ala Trp Tyr Gln Gln Thr Gln Gly Lys Ser Pro Gln Leu  
195 200 205

Leu Val Tyr Ser Ala Lys Thr Leu Ala Asp Gly Val Pro Ser Arg Phe  
210 215 220

Ser Gly Ser Gly Ser Gly Thr Gln Tyr Ser Leu Lys Ile Asn Ser Leu  
225 230 235 240

Gln Pro Glu Asp Phe Gly Ser Tyr Tyr Cys Gln His Phe Trp Ser Thr  
245 250 255

Pro Tyr Thr Phe Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Leu  
260 265 270

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His  
275 280 285

His His His His His  
290